FINAL REPORT

Role of Acidophilic Methanotrophs in Long Term Natural Attenuation of cVOCs in Low pH Aquifers

SERDP Project ER-2531

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14. ABSTRACT

The main focus of this Limited Scope SERDP project was to determine the extent to which methanotrophs may contribute to the biodegradation of chlorinated volatile organic compounds (cVOCs) in low pH groundwater aquifers. Our objectives included (1) determining whether methanotrophs exist in acidic aquifers and are capable of degrading methane and cVOCs and (2) to identity of the key methanotrophs and/or methane-oxidizing genes present in acidic groundwater systems using advanced molecular techniques. Overall, this project showed that there is a phylogenetically diverse of group of methanotrophs present in acidic groundwater aquifers, and that these organisms can potentially be important in degradation of cVOCs, including trichloroethene (TCE). There is very little research on this group of organisms in groundwater, their potential contribution to natural attenuation of cVOCs, or the possibility of enhancing their activity.

15. SUBJECT TERMS

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ACRONYM LIST

AFB – Air force base
BD – Buoyant density
BE-3R – Enrichment culture capable of growing on ethane
BGS – Below ground surface
C1 – Column 1
C2 – Column 2
C3 – Column 3
^{13}C-SIP – ^{13}Carbon stable isotope probing
CB&I – CB&I Federal Services
CF - Chloroform
CH_4 – Methane
$^{13}CH_4$ – ^{13}C-labeled methane
Cis-DCE – 1,2-*cis*-dichloroethene
Cm - Centimeter
COC – Contaminant of concern
CsCl – Cesium chloride
CT – Carbon tetrachloride
Cu – Copper
cVOCs – Chlorinated volatile organic compounds
1,1-DCA – 1,2-Dichloroethane
1,2-DCA – 1,2-Dichloroethane
DCM – 1,2-Dichloromethane
DNA – Deoxyribonucleic acid
^{13}C-DNA – ^{13}C- Deoxyribonucleic acid
^{13}C-DNA – ^{12}C- Deoxyribonucleic acid
DO – Dissolved oxygen
DoD – Department of Defense
EDB – 1,2-Dibromoethane
EPA/USEPA – Environmental Protection Agency
ESTCP – Environmental Security Technology Certification Program
ft – Foot/feet
GC-MS – Gas chromatography – mass spectrometry
Hrs – Hours
HRT – Hydraulic residence time
IH57 – Enrichment culture form IHDIV samples
IHDIV – Indian Head Division Naval Surface Warfare Center
JBMDL – Joint Base Dix-McGuire-Lakehurst
KH_2PO_4 – Potassium phosphate
KNO_3 – Potassium nitrate
L - Liter
LB – Luria Bertani broth
MG/L – Milligrams per liter
MCL – Maximum contaminant level
Min – Minute
mmoX – Soluble methane monooxygenase component A gene
MNA – Monitored natural attenuation
NAS – Naval air station
NDMA – *N*-Nitrosodimethylamine
NSWC – Naval surface warfare center
O&M – Operation and maintenance

ORP – Oxidation-reduction potential
PCE – 1,1,2,2-tetrachloroethene
PCR – Polymerase chain reaction
pMMO – Particulate methane monooxygenase
pmoA – particulate methane monooxygenase subunit A gene
ppb – Parts per billion; µg/L
r^2 – Correlation coefficient
RPM – Rotations per minute
16S rRNA – 16S ribosomal ribonucleic acid
SDC-9 – Dehalogenating consortium
SERDP – Strategic Environmental Research and Development Program
SIP – Stable isotope probing
sMMO – Soluble methane monooxygenase
1,1,1-TCA – 1,1,1,-trichloroethane
TCE – Trichloroethene
Trans-DCE – *Trans*-1,2-dichloroethene
t-RFs – Terminal restriction fragments
t-RFLP – Terminal restriction fragment length polymorphism
µL - Microliter
US – United States
USACE – United States Army Corps of Engineers
USGS – United States Geological Survey
VC – Vinyl chloride

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ABSTRACT

Objective: The main focus of this *Limited Scope SERDP project* was to determine the extent to which methanotrophs may contribute to the biodegradation of chlorinated volatile organic compounds (cVOCs) in low pH groundwater aquifers. Our objectives included (1) determining whether methanotrophs exist in acidic groundwater aquifers and are capable of degrading methane and cVOCs, with a specific focus on trichloroethene (TCE); and (2) to identity of the key methanotrophs and/or methane-oxidizing genes present in acidic groundwater systems using advanced molecular techniques, including stable isotope probing (SIP). The role of methanotrophs in biodegrading cVOCs in low pH groundwater has received little study as has the potential to enhance their activity with methane or other amendments.

Technical Approach: Aquifer samples were obtained from three DoD sites (including two different aquifer formations at one site) with low pH groundwater. Microcosms were initially prepared from each site to determine whether the addition of methane and air into the samples stimulated native methanotrophs to biodegrade TCE. Ethane and ethene were also tested as cometabolic substrates at one of the sites. Degradation rates were determined where applicable, and samples from active microcosms were added to mineral salts media with methane as a sole substrate to enrich and isolate native acidophilic methanotrophs. Enrichments with ethane or ethene were also prepared from some samples. Characterization of the methanotrophic communities in the acidic aquifers was conducted using stable isotope probing (SIP) and probing for *pmo*A and *mmo*X genes in samples, which code for the two common enzymes (particulate methane monooxygenase; pMMO and soluble methane monooxygenase; sMMO) used by methanotrophs to convert methane to methanol (and also to oxidize cVOCs). For SIP analysis, ^{13}C-methane ($^{13}CH_4$) was added to active bottles, and organisms that degraded the $^{13}CH_4$ were identified based upon ^{13}C enrichment in DNA. Finally, a column study was performed using site materials from one location to assess methane and cVOC degradation in a more realistic flow-through system.

Results: Methane consumption and degradation of cVOCs was observed in acidic aquifer samples collected from all three DoD sites. Interestingly, at one site where samples were collected from two different aquifer formations, methane and cVOC degradation was observed in one formation but not the other, possibly due to the anaerobic conditions in the latter formation where oxygen loss was evident (probably via abiotic oxidation of reduced minerals), but methane was not consumed. Methanotrophic enrichment cultures capable of degrading TCE and a number of other cVOCs at pH 4 were isolated from two of the three sites. Cultures capable of degrading cVOCs at pH 4 after growth on ethane or ethene also were enriched. Although degradation of only a small subset of chlorinated ethenes and ethanes by the various enrichment cultures was evaluated, the data indicate differences in the cVOCs degraded among the acidophilic enrichment cultures and between some of these cultures and the more widely studied neutrophilic methanotrophs. Further study is warranted to better understand the selectivity of the presumptive sMMO or pMMO enzymes catalyzing reactions under acidic conditions.

Stable isotope probing (SIP) of active microcosms from the two sites tested showed that a variety of methanotrophs incorporated ^{13}C into their DNA (from added $^{13}CH_4$). A total of thirty five 16S rRNA clones were derived and identified. The clones clustered largely in the *Alphaproteobacteria* and

Gammaproteobacteria but were not closely related to known acidophilic methanotrophs. The results are unexpected but interesting, suggesting that many methanotrophs are present in the acidic groundwater and that they are more phylogenetically diverse than previously reported. Future work is needed to isolate these acidophilic methanotrophs to better understanding their abundance and role in biodegradation of cVOCs and other contaminants at acidic groundwater sites.

Benefits: Overall, this project showed that a diversity of acidophilic methanotrophs are present in low-pH groundwater aquifers, and that these organisms can potentially be important in degradation of TCE and other cVOCs. There is very little research on this group of organisms in low pH groundwater, their potential contribution to natural attenuation of cVOCs, or the possibility of enhancing their activity. In that reductive dechlorination does not generally occur at low pH, aerobic cometabolism may be particularly important as a remedial mechanism for cVOCs. The presence of organisms capable of growing at low pH on ethane or ethene and degrading cVOCs was also indicated based on microcosm and enrichment culture data. The potential for these cometabolic substrates (which are often formed during reductive dechlorination of cVOCs) to stimulate also has received little study to date.

Based on the initial results of this Limited Scope Study, there are several areas that we believe warrant additional investigation including (1) measuring the activity and degradation kinetics of acidophilic methanotrophs at low methane and cVOC concentrations as may be observed in dilute cVOC plumes that are problematic for DoD; (2) assessing the potential for acidophilic methanotrophs from groundwater to biodegrade cVOCs after growth on substrates other than methane (e.g., small fatty acids, alcohols), as several methanotrophs recently been described that are facultative; (3) further assessing the suite of cVOCs that may be susceptible to degradation by acidophilic methanotrophs, to include chlorinated methanes, ethanes, ethenes, and whether activity is culture-specific; (4) evaluating key factors contributing to differences in cVOC degradation among sites and/or strains (e.g., effect of dissolved metals or other co-contaminants, extent of substrate inhibition, pH optima and extremes); and (5) more clearly identifying the types of MMO enzymes that are active at low pH, determining how they differ from those of neutrophilic bacteria, and developing appropriate primer sets to detect and quantify them via qPCR as a measure of degradative potential in acidic aquifers.

1.0 PROJECT BACKGROUND AND OBJECTIVES

1.1 Technical Objective

The main focus of this research effort was to determine the extent to which "acidophilic" methanotrophs may contribute to the biodegradation of chlorinated volatile organic compounds (cVOCs) in low pH groundwater aquifers. For the purposes of this project, an "acidophilic methanotroph" will be defined as generally as capable of growth at pH 5 or below, with the understanding that these organisms are not necessarily "obligate acidophiles" that require highly acidic pH to survive. These definitions are consistent with Madigan et al., (1997). Our objectives included (1) determining whether methanotrophs exist in acidic groundwater aquifers and are capable of degrading methane and cVOCs, with a specific focus on trichloroethene (TCE); and (2) to identity of the key methanotrophs and/or methane-oxidizing genes present in acidic groundwater systems using advanced molecular techniques, including stable isotope probing (SIP). Because aerobic degradation of TCE via cometabolism produces no daughter products that are easily identified in groundwater (**Figure 1**), this process is difficult to assess *in situ*, and may play a much more important role in natural attenuation than currently thought. Moreover, the potential for biostimulating these populations to enhance cVOC degradation in low pH aquifers with methane or possibly other organic substrates also has received little consideration.

1.2 Background

Chlorinated volatile organic compounds represent one of the largest remediation challenges and costs at commercial and military sites in the U.S. (e.g., National Research Council, 2013). Anaerobic bioremediation via carbon source addition (with or without bioaugmentation with dechlorinating bacteria) is a commonly used approach to treat cVOCs *in situ* (Stroo and Ward, 2010). However, one significant issue with this technology is that reductive dechlorination processes are typically inhibited at pH values < ~ 5.5 (e.g., **Figure 2**; Vainberg et al., 2009). Aquifer buffering has been attempted in some instances (e.g., Hatzinger et. al., 2006; Schaefer et al., 2010), but the amount of buffer required makes this process cost prohibitive for other than very small sites.

Low pH groundwater is common throughout the Northern Atlantic Coastal Plain aquifer system in the U.S, which runs south from Long Island, New York through much of North Carolina (Denver et al., 2015). In fact, of 419 samples for which pH data were available from a USGS water quality study of this aquifer system, 250 (60%) were reported to have pH values of 5.5 or below (data archive; https://pubs.usgs.gov/circ/1353/). This aquifer system, which includes a number of large military facilities and some large urban areas, is also significantly impacted by cVOCs (Denver et al., 2015). Some of the affected military sites with plumes of cVOCs in acidic groundwater include Joint Base Dix-McGuire-Lakehurst (JBDML; NJ), Raritan Arsenal (NJ), Indian Head Division Naval Surface Warfare Center (IHDIV; MD), and Marine Corps Base Quantico (VA) among others. In the absence of pH adjustment, reductive dechlorination is not anticipated to be an effective treatment process at these low pH sites.

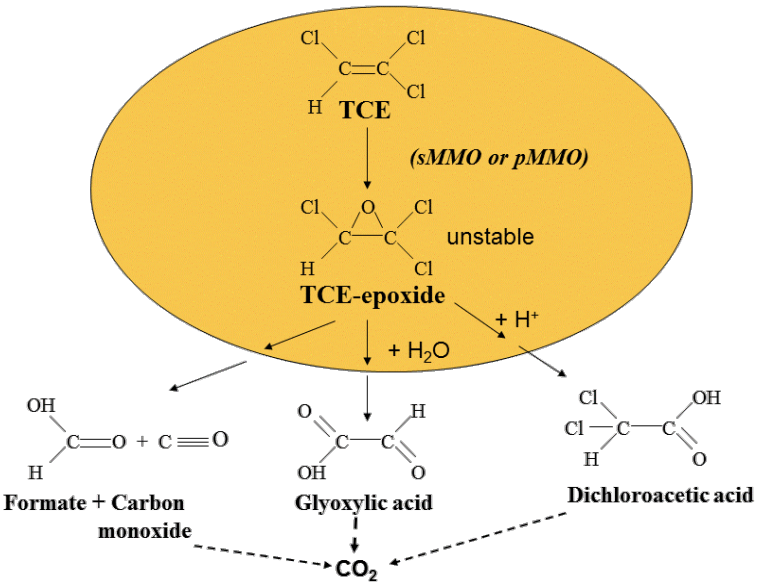


Figure 1. Pathways of TCE degradation during aerobic cometabolism. (pathways derived from Little et al., 1998)

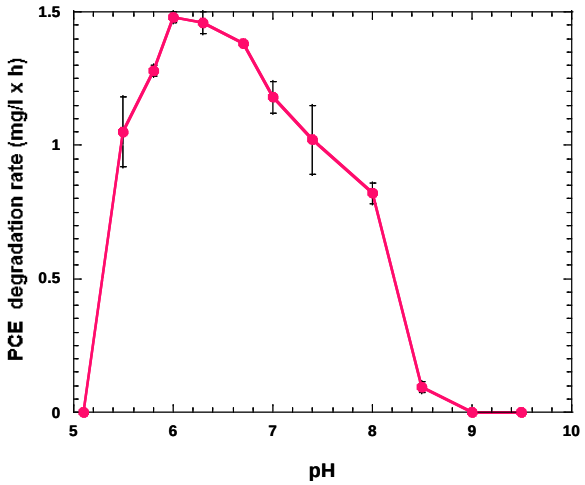


Figure 2. Effect of pH on reductive dehalogenation of PCE by the *Dehalococcoides* culture SDC-9. Dehalogenation is completely inhibited at pH 5.

The studies of methanotrophs and their applications for pollutant biodegradation are not new pursuits. Methanotrophs were first identified in 1906 (Söhngen, 1906), although it was not until the 1970s and 1980s that they began to receive serious study from a physiological, taxonomic and environmental perspective (e.g., Whittenbury et al., 1970; Takeda et al., 1976; Colby et al., 1977; Green and Dalton,

1985). The observation that methanotrophs could aerobically biodegrade TCE (Oldenhuis et al., 1989; Wilson and Wilson, 1985), subsequently led to significant laboratory research in this area and field trials where methane was applied *in situ* to stimulate pollutant biodegradation (Semprini et al., 1990, 1991; Pfiffner et al., 1997). However, there is comparatively little research on the impact of indigenous methanogenic populations on the natural attenuation of cVOCs (Wymore et al., 2007), and a vast majority of the methanotrophs that have been the subject of biodegradation research to date grow most effectively at neutral pH and, with a few recent exceptions (Danilova et al., 2013), are incapable of growth below pH 5 (Semrau et al., 2010; Dedysh et al., 1998a,b). **As such, the importance of the methanotrophs in the attenuation of pollutants in acidic aquifers has received little study.**

Two new groups of acidophilic methanotrophs have been discovered over the past fifteen years. The first group, which are *Alphaproteobacteria* in the genera *Methylocella*, *Methylocapsa*, and *Methylocystis*, are all capable of growth below pH 5, and, unlike other methanotrophs described to date, many are capable of growing on substrates such as ethanol and acetate in addition to single carbon compounds (i.e., facultative methanotrophs) (Dedysh et al., 1998a,b, 2000, 2002, 2004; Kip et al., 2011; Im et al., 2011; Belova et al., 2011; Im and Semrau, 2011). Moreover, some of these organisms constitutively express particulate methane monooxygenase (pMMO) during growth on these alternate substrates (Yoon et al., 2011; Belova et al., 2011). Particulate and soluble methane monooxygenase (sMMO) are enzymes that convert methane to methanol in typical methanotrophs, and they are also the enzymes that are known to catalyze the degradation of a variety of chlorinated solvents (e.g., Oldenhuis et al., 1989; Fox et al., 1990; Dispirito et al., 1992; Koh et al., 1993; Hanson and Hanson, 1996; Lontho and Semrau, 1998; Lee et al., 2006). The second group of recently discovered methanotrophs are *Verrucomicrobia*, a unique phylum of organisms that may constitute > 10% of all soil bacteria, and also occur in aquatic, marine, groundwater, and other common environments, yet whose ecological roles are largely unknown (Sangwan et al., 2005; Wagner and Horn, 2006; Semrau, 2011; Hao et al., 2009). The methanotrophic *Verrucomicrobia*, which have been placed in the genus *Methylacidiphilum*, are by far the most acidophilic methanotrophs reported to date, with pH optima between 2 and 2.5, and the ability to grow at pH values as low as 0.8 (Dunfield et al., 2007; Pol et al., 2007; Hou et al., 2008).

There are only a few reports concerning the ability of the acidophilic methanotrophs to biodegrade pollutants, and these are primarily focused on the bacterium *Methylocystis* strain SB2 (Jagadevan and Semrau, 2013; Im and Samrau, 2011; Yoon et al., 2011). This organism, which has only pMMO, was observed to degrade TCE, VC, *trans*-1,2-dichloroethene (*trans*-DCE), and 1,1,1-trichloroethane (1,1,1-TCA) after growth on methane, ethanol, or acetate. The ability of this strain and potentially other "facultative methanotrophs" to utilize substrates other than methane for growth and to couple this metabolism to cVOC degradation is important because alternate substrates, such as acetate and other fatty acids, can occur in groundwater aquifers under a variety of conditions, and along with methane, may naturally support cVOC degradation by these organisms. There is very little information in this area.

We recently evaluated the ability of the acidophilic strain, *Methylocella palustris* to degrade TCE and several other cVOCs. Unlike *Methylocystis* strain SB2, which only possesses pMMO, this organism only has genes encoding for sMMO (Dedysh et al., 2000). However, like the *Methylocystis* spp., *Methylocella* spp. have been observed to grow on a variety of compounds other than methane, including acetate,

pyruvate, succinate, malate, and ethanol (Dedysh et al., 2005). This genus can also fix atmospheric nitrogen as a N source for growth (Dedysh et al., 2005). In our study, *Methylocella palustris* rapidly degraded TCE at pH values as low as 4 (**Figure 3**), and was also observed to oxidize a number of other important cVOCs, including *cis*-DCE, VC, 1,2-dichloroethane (1,2-DCA), dichloromethane (DCM), 1,2-dibromoethane (EDB), and chloroform (CF), but not 1,1,2,2,-tetrachloroethene (PCE) or 1,1,1, trichloroethane (TCA) (**Figure 4**). To our knowledge, this is the first report of VOC degradation by *Methylocella* spp. The data indicate that this ability is potentially widespread among the acidophilic methanotrophs.

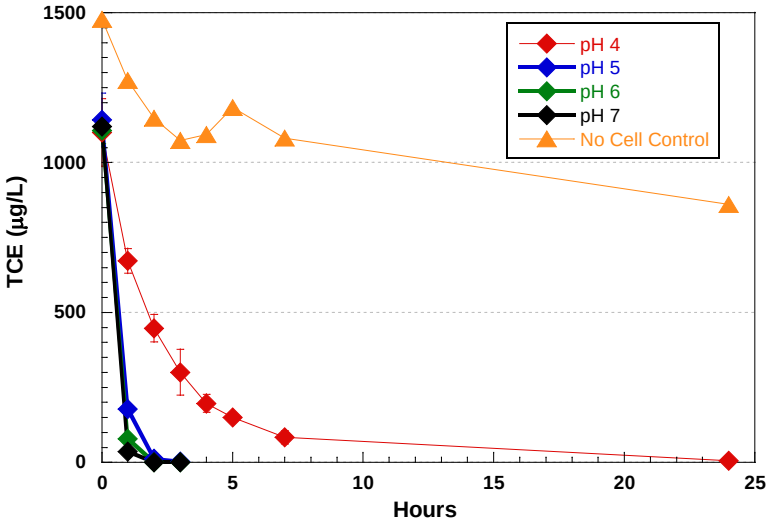


Figure 3. Effect of pH on degradation of TCE by *Methylocella palustris*. The bacterium degraded more than 1 mg/L of TCE in 24 h at pH 4 (*Hatzinger et al., unpublished data*).

At present, there is little information about the occurrence or biodegradative activity of acidophilic methanotrophs of any groundwater aquifers, as most studies to date have been with pure cultures isolated primarily from peat ecosystems (*Alphaproteobacteria*) or geothermal areas (*Verrucomicrobia*). However, much like groundwater, peat bogs are nutrient poor environments, and the methanotrophs isolated from them tend to grow well only in dilute, oligtrophic media (Kip et al., 2011; Dedysh et al., 2002). It is likely that these organisms or similar ones may exist in low pH aquifers, since methanotrophs represent a very diverse and nearly ubiquitous class of bacteria, occurring nearly everywhere methane and oxygen exist together (Semrau, 2011). The genus *Methylocella*, for example, which was originally isolated from an acid peat bog, has recently been shown to be much more widely distributed in the environment than previously thought, although aquifers were not assessed (Rahman et al., 2011). Moreover, some acidic aquifers, such as that underlying Fort Dix (NJ) have interbedded peat layers, which serve as a continuing source of TCE in contaminated regions of the aquifer, and as a potential site for growth and activity of acidophilic methanotrophs. In addition, while acid tolerant *Methylacidiphilum* has yet to be reported in groundwater aquifers (perhaps because no studies have been performed), organisms of the phylum *Verrucomicrobia* have been detected in large numbers in groundwater, including 6.7 % of the microbial community in an oil contaminated groundwater sample (Hao et al., 2009).

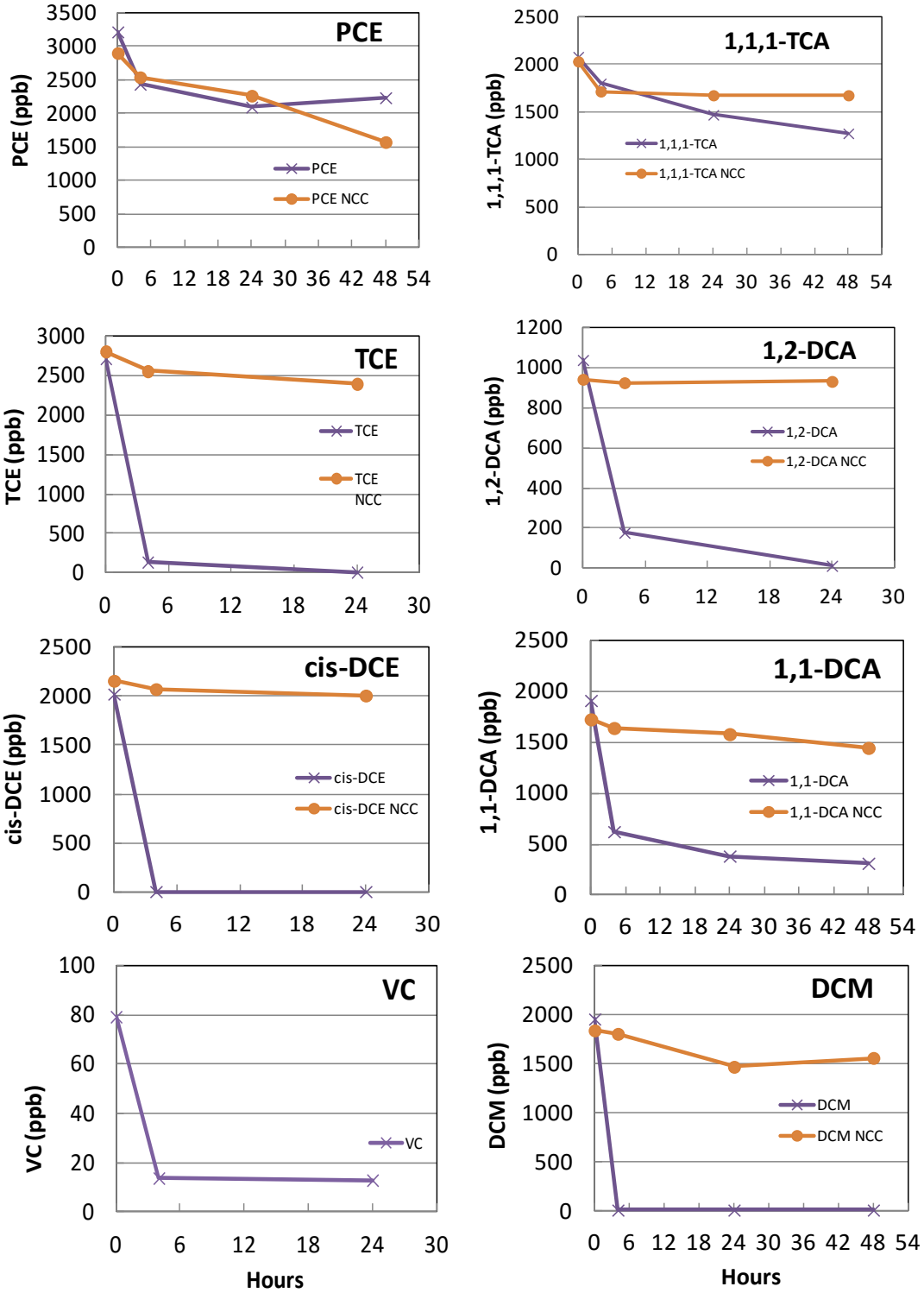


Figure 4. Biodegradation of various cVOCs by *Methylocella palustris.* Cells were grow on methane, washed, and placed in vials with the various compounds without additional substrate. Values are means from duplicate samples. NCC = No cell control. The control sample for VC could not be analyzed. Graphs for CT (no degradation), 1,4-dioxane (no degradation), 1,2-dibromoethane (degradation) and 1,1-dichloroethene (degradation) are not plotted.

To examine the potential occurrence of acidophilic methanotrophs in groundwater, we prepared microcosms from a location at former Myrtle Beach AFB, South Carolina, with natural pH values between 3.3 to 4.1. These microcosms received methane or propane gas in air and inorganic nutrients. The original goal of this study was to evaluate the potential for 1,4-dioxane degradation, which did not occur. However, over several weeks of incubation, during which time the pH in the bottles declined to as low as 2, the consumption of methane (but not propane) was observed (**Figure 5**). Subsequently, a mixed culture was successfully enriched from the site at pH 4 on methane as a sole carbon source. This culture was observed to degrade TCE, VC, and cis-DCE (but not 1,4-dioxane) at pH 4 (**Figure 6**). ***This is the first observation, to our knowledge, that native methanotrophs in an extremely acidic groundwater environment may be active in cVOC degradation. The identities of the organisms in this mixed culture are not yet known.***

One additional important aspect of cometabolism for long term natural attenuation of cVOCs is the fact that the degradative organisms do not rely on the target cVOC for growth, and thus can potentially degrade the contaminant to very low concentrations. This principle was recently proven in our laboratory for cometabolic degradation of N-nitrosodimethylamine (NDMA), which was biodegraded from µg/L to low ng/L concentrations *in situ*, *ex situ*, and by a pure culture of a propanotrophic bacterium (**Figure 7**; Fournier et al, 2009; Hatzinger et al., 2011). Similarly, we recently showed that ethane-degrading bacteria were capable of degrading 1,2-dibromoethane (EDB) to < 0.02 µg/L (**Figure 8;** Hatzinger et al., 2015). With dilute plumes of cVOCs in groundwater, proving that bacteria degrade a few µg/L of a contaminant to ng/L concentrations is important if biodegradation is to play an important role in monitored natural attenuation (MNA).

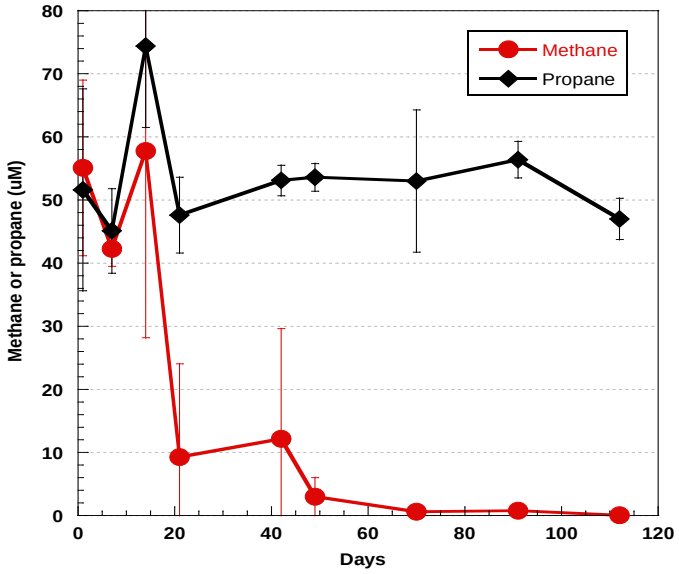


Figure 5. Biodegradation of methane, but not propane, in aerobic microcosms prepared from a low pH site at Myrtle Beach AFB. The starting pH of these microcosms was ~ 3.8 and declined to ~ 2.0 with time. An enrichment culture capable of degrading methane at pH 4 was subsequently isolated from these samples (see Figure 4).

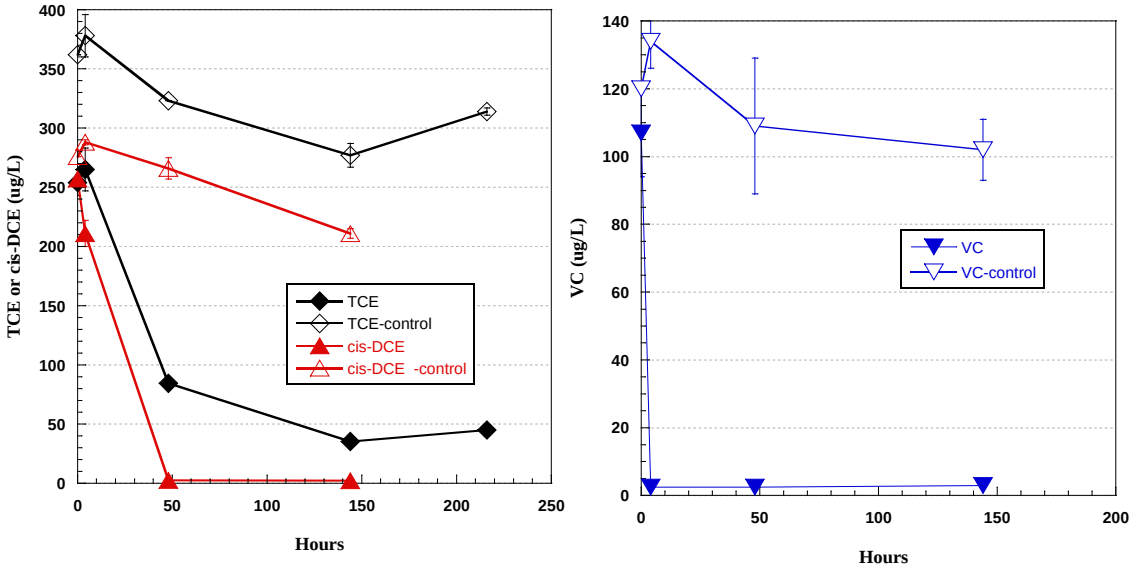


Figure 6. Biodegradation of TCE, *cis*-DCE (left panel) and VC (right panel) by an enrichment culture of methanotrophs from former Myrtle Beach AFB. The pH of these enrichments was 4.0. No cell controls are also provided. Data are means and standard deviations from duplicate samples and error bars were generally smaller than symbols.

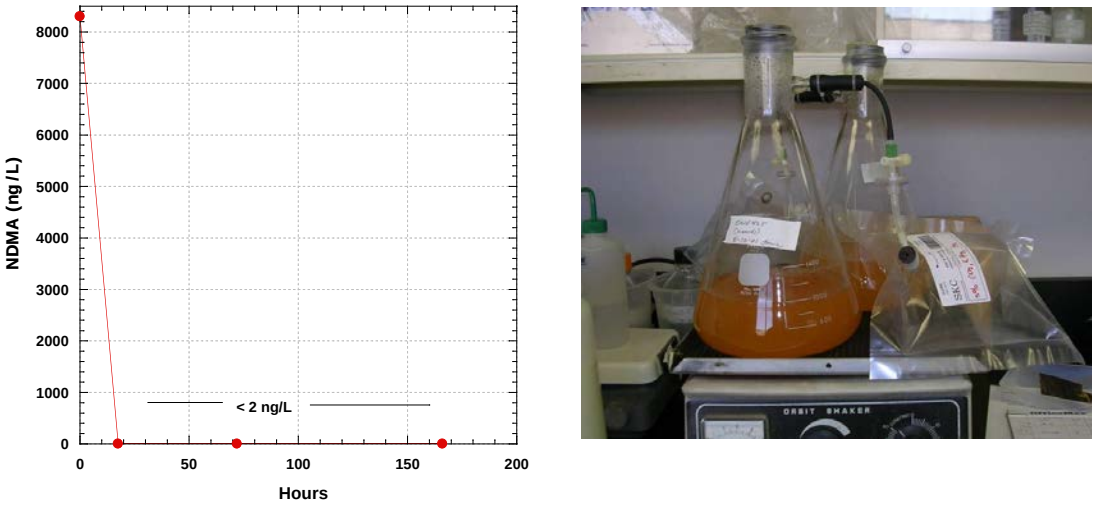


Figure 7. Cometabolic biodegradation of NDMA by the propanotroph *Rhodococcus ruber* ENV425 (left panel). Cells were grow at room temperature with propane in the headspace (right panel), and NDMA was biodegraded to < 2 ng/L within 18 h. Data from Fournier et al., (2009).

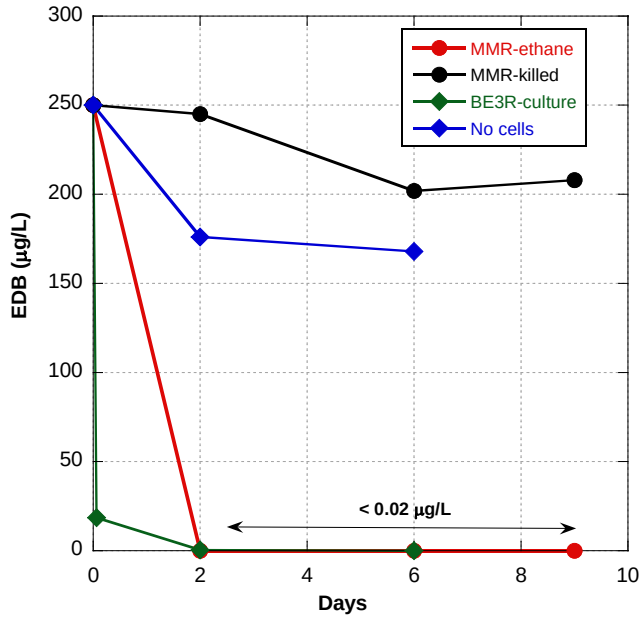


Figure 8. Biodegradation of 1,2-dibromoethane (EDB) in ethane-fed microcosms from the FS-12 groundwater plume at MMR and by an enrichment culture isolated on ethane gas from the same aquifer (BE-3R). The MMR microcosms were pre-incubated with ethane, oxygen and nutrients for several weeks prior to introduction of EDB. Killed controls or microcosms not receiving ethane showed no EDB degradation. From Hatzinger et al., (2015).

The primary purpose of this *Limited Scope SERDP project* was to document the presence of acidophilic methanotrophs in low pH aquifers, to characterize the methanotrophs/enzymes that occur, and to assess the extent to which they may contribute to long-term degradation of chlorinated ethenes in low pH groundwater as a natural attenuation mechanism. Methane, and short chain fatty acids (which some of these methanotrophs can use) routinely occur in groundwater and within chlorinated solvent plumes through natural (e.g., emanating from low conductivity formations) or man-made (e.g., in plumes originating in landfills with cVOCs, after degradation of co-contaminants with cVOCs, or via addition of organic substrates for anaerobic cVOC remediation in source zones) processes. In addition, only low concentrations of these substrates may be required for microbial activity based on the oligotrophic nature of the acidophilic methanotrophs described to date. Thus, conditions may be suitable in many low pH aquifers to support their growth and degradative activity. We also conducted limited studies with ethane and ethene in some of the site samples to assess the extent to which these gases may support cVOC degradation in acidic groundwater.

1.3 Research Questions

Our initial laboratory studies from former Myrtle Beach AFB suggest that acidophilic methanotrophs can exist in low pH aquifers (pH < 4), and that these organisms may contribute to cVOC degradation in these environments. However, other than this experiment, there is very little data on the presence or activity of these organisms in acidic groundwater. Key questions addressed during this *Limited Scope SERDP Project* were as follows:

- Are known acidophilic methanotrophs capable of cVOC biodegradation typically present in low pH aquifers?
- Are sMMO, pMMO or a variant of these enzymes detectable in low pH aquifers?
- What are the identities of active methanotrophs in low pH aquifers – are they pH-tolerant species of widely studied methanotrophs (e.g., *Methylobacter*) or members of newly discovered genera or phyla (e.g., *Methylocella* or *Verrucomicrobia*)?
- Do these methanotrophs degrade TCE, *cis*-DCE and/or VC *in situ*?

1.4 Research Tasks

Research for this project was divided into four technical tasks.

Task 1. Site Selection and Sample Collection. Samples were obtained from three DoD sites with TCE plumes and low groundwater pH. Soil and groundwater collected from the sites were used in Task 2, Task 3, and Task 4 testing.

Task 2. Microcosm Studies and Microbial Enrichments. Microcosms were initially prepared from each Task 1 site to determine whether the addition of methane and air into the samples stimulated native acidophilic methanotrophs to biodegrade TCE. In some instances ethane and ethene were also tested. ^{13}C-Methane was added to some samples to facilitate identification of active methanotrophs via stable isotope probing (SIP) analysis in Task 3. Degradation rates were determined where applicable. Enrichments were then performed from the most active bottles to isolate native acidophilic methanotrophs and ethanotrophs.

Task 3. Microbial Community Characterization and SIP Analysis. Characterization of the methanotrophic communities in the acidic aquifers was conducted using SIP analysis and probing for *pmo*A and *mmo*X genes in samples, which code for the two common enzymes used by methanotrophs to convert methane to methanol (and also to oxidize cVOCs). The SIP analysis, was conducted with batch microcosm samples and enrichments.

Task 4. VOC Degradation by Acidophilic Methanotrophs in Simulated Flow-Through Aquifers. A laboratory column study was performed using site materials from one location identified in Task 1, to assess VOC degradation kinetics in the acidic aquifer under methanotrophic conditions.

2.0 MATERIALS AND METHODS

2.1 Site Evaluation and Sample Collection

Aquifer samples were collected from three sites with naturally acidic groundwater pH for use in microcosm studies: (1) Joint Base McGuire-Dix-Lakehurst (JBMDL; NJ); (2) Indian Head Division Naval Surface Warfare Center (IHDIV; MD), and (3) Naval Surface Warfare Center, Dahlgren (NSWC, Dahlgren; VA). Aquifer samples at the first two sites were collected using a direct-push (i.e., Geoprobe) rig, and the third site was sampled via hand-auger. Groundwater was obtained from an existing local well via low-flow sampling (Puls and Barcelona, 2005) at each location. Additional details on sample collection and characteristics from each location are provided in Section 2.2.

2.2 Microcosm Studies

Microcosms were prepared using aquifer solids and groundwater collected during Task 1. The microcosms received varying concentrations of methane in air, and selected treatments received inorganic nutrients as detailed for each experiment. Appropriate live (i.e, no methane) and killed controls were also prepared. In some experiments, treatments with ethane or ethene were evaluated. Bottles were spiked with TCE and incubated at 15 ^{o}C with gentle shaking. Methane (or other gases) and oxygen were monitored at least bi-weekly by headspace gas chromatography (GC) analysis to evaluate microbial activity. When headspace oxygen concentrations declined to less than levels found in ambient air (i.e., less than 21%), additional oxygen was added to restore the microcosm headspace to approximately 20% oxygen. Evaluation of TCE degradation over time was performed using gas chromatography-mass spectrometry (GC-MS; EPA Method 8260).

2.2.1 Joint Base McGuire-Dix-Lakehurst (JBMDL)

Microcosm tests were performed using site materials collected from the MAG-1 Area at former McGuire AFB, now a component of JBMDL. The MAG-1 aquifer has TCE in groundwater from a former parts washing operation. The natural groundwater pH in this region of the aquifer is $\leq$ 4. Aquifer solids were collected by direct push (Geoprobe) from the Kirkwood formation (7-15 ft below ground surface; BGS) and from the Vincentown formation (25-30 ft BGS), which underlies the Kirkwood formation **(Figure 9**). The aquifer materials were logged by a certified geologist, and samples were transferred in the field from acetate sleeves to 1L glass screw-cap jars. Site groundwater was collected from well MAG-204 using low-flow sampling. Aquifer solids and groundwater were transported on ice to the CB&I Laboratory in Lawrenceville, NJ, where they was stored at 4 ^{o}C until use.

Microcosms were prepared in 160 mL glass serum bottles with Teflon-lined septa. The aquifer solids from each formation were homogenized, and then 25 g portions were added to each serum bottle along with 85 mL of site groundwater. The specific treatments prepared were as follows:

Treatment 1: KILLED CONTROLS – These treatments were amended with mercuric chloride solution to a final concentration in groundwater of approximately 300 mg/L to inhibit microbial activity, and were used to evaluate abiotic loss of methane, oxygen, and cVOCs;

Treatment 2: METHANE + NUTRIENTS– These treatments received 10% methane gas in the headspace (balance room air) and inorganic nutrients (10 mg/L N from KNO_3 and 10 mg/L P from KH_2PO_4);

Treatment 3: METHANE + TCE + NUTRIENTS – These treatments received 10% methane gas in the headspace (balance room air), TCE (final aqueous concentration of approximately 500 μg/L) and inorganic nutrients (i.e., nitrate and phosphate).

Six replicates were prepared for each treatment. Initially, microcosms were prepared using standard ^{12}C-methane. Microcosms were incubated shaking on their sides in the dark at 15 ^{o}C. To prevent the loss of the added alkane gases during sampling, samples were removed at selected time points through the septa using Gastight glass syringes and analyzed for cVOCs via EPA Method 8260B (groundwater), oxygen, and reduced gases (headspace gas) via EPA Method 3810, RSK-175. pH was analyzed using a meter and probe or pH test strips. The liquid volume removed was replaced with sterile-filtered room air so that a vacuum was not created. Microcosms constructed using soils from the Kirkwood formation had an initial pH of 3.4, and microcosms constructed using soils from the Vincentown formation had an initial pH of 4.4.

On days 20 and 25, following the depletion of methane in the Kirkwood microcosms, additional methane was added to restore the microcosms to 10% ^{12}C-methane in the headspace. On days 33 and 36, when the methane was again depleted, additional methane was again added, however, three of the six replicates of each treatment received ^{13}C-labeled methane (99 atom %; Sigma-Aldrich, Milwaukee, WI), while the remaining three replicates continued to receive ^{12}C-methane. In addition to monitoring TCE, oxygen, and methane concentrations, following the addition of ^{13}C-labeled methane, samples of the microcosm slurry (typically 25-30 mL) were removed through the septa, frozen at -20 ^{o}C, and analyzed to determine which bacteria incorporated the ^{13}C-methane via Stable Isotope Probing (SIP) (See Section 2.4).

Figure 9. Collection of aquifer cores and groundwater samples from the MAG-1 Area of McGuire AFB at JBMDL.

2.2.2 Naval Surface Warfare Center Dahlgren, VA (NSWC Dahlgren)

Site soil and groundwater were collected from the Churchill Range at NSWC Dahlgren. This site is not contaminated with cVOCs, but is known to have low groundwater pH. Saturated aquifer material was collected from a depth of 4.5-5.5 ft BGS using a hand auger, and placed in 1L glass jars. Groundwater was collected into 1L cleaned bottle from well MW-10, which was emplaced during ESTCP Project ER-201028 (https://serdp-estcp.org/Program-Areas/Environmental-Restoration/Contaminated-Groundwater/Emerging-Issues/ER-201028/ER-201028). Aquifer solids and groundwater samples were delivered to CB&I's laboratory in Lawrenceville, New Jersey in coolers with ice, and were stored at 4 °C until initiation of the microcosm studies. Aquifer solids were homogenized by hand using a modified cone-and-quarter technique. Microcosms were prepared in 160 mL serum bottles by adding 25 g soil and 85 mL groundwater, as described for the JBMDL samples. All bottles were sealed with Teflon-lined butyl rubber septa and aluminum crimp caps.

Treatments were prepared in duplicate for each set as follows:

Treatment 1: HIGH METHANE + NUTRIENTS – These treatments received 10% methane gas in the headspace (balance room air) and inorganic nutrients (10 mg/L N from KNO_3 and 10 mg/L P from KH_2PO_4);

Treatment 2: MEDIUM METHANE ± NUTRIENTS – These treatments received 1% methane gas in the headspace (balance room air) with or without inorganic nutrients;

Treatment 3: LOW METHANE ± NUTRIENTS – These treatments received 0.1% methane gas in the headspace (balance room air) with or without inorganic nutrients;

Treatment 4: NUTRIENTS ONLY – These treatments received inorganic nutrients only;

Treatment 5: ETHANE + NUTRIENTS – These treatments received 1% ethane gas in the headspace (balance room air) and inorganic nutrients;

Treatment 6: ETHENE + NUTRIENTS – These treatments received 1% ethene gas in the headspace (balance room air) and inorganic nutrients;

Treatment 7: KILLED CONTROLS – These treatments received mercuric chloride (final aqueous concentration of 500 mg/L) to inhibit microbial activity. These treatments also received 1% methane, 1% ethane, and 1% ethene gases in the headspace (balance room air).

Microcosms were incubated shaking on their sides in the dark at 15 ^{o}C. To prevent the loss of the added alkane gases during sampling, samples were removed at selected time points through the septa using Gastight glass syringes and analyzed for cVOCs, oxygen, reduced gases, and pH as previously described.

Following the completion of this initial study, a second study using the same site materials from NSWC, Dahlgren was performed with the express intention of adding ^{13}C-methane and analyzing the microbial community via SIP.

SIP-specific microcosms were set up as follows:

Treatment 1: KILLED CONTROLS – These treatments were amended with mercuric chloride solution (final concentration in groundwater approximately 300 mg/L) to inhibit microbial activity, and were used to evaluate abiotic loss of methane and cVOCs;

Treatment 2: METHANE + NUTRIENTS – These treatments received 10% methane gas in the headspace (balance room air) and inorganic nutrients (10 mg/L N from KNO_3 and 10 mg/L P from KH_2PO_4);

Treatment 3: METHANE + TCE + NUTRIENTS – These treatments received 10% methane gas in the headspace (balance room air), TCE (final aqueous concentration of approximately 500 μg/L) and inorganic nutrients.

Six replicates were prepared for each treatment. Microcosms were prepared initially using ^{12}C-methane and were incubated and sampled as previously described. On day 54, following the depletion of methane, additional methane was added to restore the microcosms to 10% ^{12}C-methane in the headspace. On day 81, when this methane was again depleted, methane was again added to 10%, however, two replicates of each treatment received ^{13}C-labeled methane, while two replicates continued to receive ^{12}C-methane. In addition to monitoring TCE, oxygen, and methane concentrations, following the addition of ^{13}C-labeled methane, samples of the microcosm slurry (typically 25-30 mL) were removed through the septa, frozen at -20^{o}C, and analyzed for which bacterial populations incorporated the ^{13}C-methane via SIP as detailed in Section 2.4.

2.2.3 Indian Head Division, Naval Surface Warfare Center, MD (IHDIV)

Water from well MW42 and soil from Area 57 was used in the preparation of the IHDIV microcosms. The soil core material was collected by hand auger from two boreholes (A2 and A6) used to install electrodes for a separate research effort. The material was collected over a 0.7 m interval starting at the top of the water table. The approximate depth ranged from ~ 2 m to 2.7 m below ground surface in the two boreholes. The saturated aquifer materials were placed in clean glass jars and shipped overnight to the CB&I Laboratory on ice along with the water samples.

Treatments were prepared in duplicate for each set as follows:

Treatment 1: HIGH METHANE + NUTRIENTS – These treatments received 10% methane gas in the headspace (balance room air) and inorganic nutrients (10 mg/L N from KNO_3 and 10 mg/L P from KH_2PO_4);

Treatment 2: MEDIUM METHANE ± NUTRIENTS – These treatments received 1% methane gas in the headspace (balance room air) with or without inorganic nutrients;

Treatment 4: ETHENE + NUTRIENTS – These treatments received 10% ethene gas in the headspace (balance room air) and inorganic nutrients (i.e., nitrate and phosphate);

Treatment 6: NUTRIENTS ONLY – These treatments received inorganic nutrients only;

Treatment 7: KILLED CONTROLS – These treatments received mercuric chloride (final aqueous concentration of 500 mg/L) to inhibit microbial activity. These treatments also received 1% methane, 1% ethane, and 1% ethene gases in the headspace (balance room air).

Microcosms were incubated shaking on their sides in the dark at 15°C and sampled as described for previous studies. SIP analysis was not performed on this set of microcosms.

2.3 Microbial Enrichments

2.3.1 Growth and Culture Conditions

Microbial enrichments on methane (and ethane or ethene for some samples) were initiated using site soils from the three locations described in Task 1. Enrichments were prepared by placing soil into a bacteriological growth medium at a ratio of 0.1 g soil per 10 mL medium in a shake flask. The following media, all adjusted to pH 4.0-4.3 with phosphoric acid, were used during enrichment culturing: ATCC Medium 2157 (*Methylocella* medium; ATCC 1994), OB3b Medium (Cornish et al., 1984), Basal Salts Medium (Hareland et al., 1975), and Nitrate Mineral Salts (NMS; Chu and Alvarez-Cohen, 1996). Methane, ethane, propane, and ethene gases were each provided in the headspace as the sole source of carbon and energy, with the balance of the headspace comprised of sterile-filtered room air. Once cultures grew turbid, they were passed at a ratio of 1 mL culture to 100 mL medium into fresh medium. After 3 passes, the cultures were tested for the ability to degrade TCE. Over 20 enrichment cultures were obtained.

2.3.2 Biodegradation Testing

Following three successful passes into fresh medium, each enrichment culture was tested for its ability to biodegrade TCE. Selected cultures were also tested for degradation of *cis*-DCE, VC, and/or other cVOCs. To test for contaminant degradation, cells were washed via centrifugation to remove any residual substrate gas, resuspended in fresh growth medium, and placed in a serum via which was then sealed with a Teflon-lined septum and aluminum crimp seal. Each vial received a spike of cVOC (generally 20 μM). Samples were taken immediately after the initial contaminant spike (i.e., time zero), and at set timepoints thereafter and analyzed for cVOCs as described previously.

2.4 Stable Isotope Probing

2.4.1 DNA Extraction and Separation

DNA in the frozen slurry samples was extracted using FastDNA spin kit for soil samples (MP Biomedical, Solon, OH). The concentrations of the extracted DNA were determined using a NanoDrop ND-1000 Spectrophotometer (Fisher Scientific., Fair Lawn, NJ). The extracted DNA was separated into ^{13}C-DNA and ^{12}C-DNA fractions using equilibrium centrifugation in CsCl density gradients as described previously (Yu and Chu, 2005) with minor modifications. Briefly, the extracted DNA (1 μg) was loaded into 3.5-mL Beckman centrifuge tubes containing 1.0 g/mL CsCl in TE buffer solution. All tubes were sealed and centrifuged using a desk-top Beckman Coulter TLX-120 Optima Ultracentrifuge in a TLA 100.3 rotor at 70,000 rpm at 20 °C for 24 h. A fraction recovery system (Beckman Coulter, CA) was used to collect ^{13}C- and ^{12}C-DNA fractions. A syringe pump (Model 7801001, Fisher Scientific, Fair Lawn, NJ) set to a flow rate 250 μL/min was used to dispense mineral oil into the top of the tube and to collect 20 fractions of 100 μL each from the bottom of the tube. The buoyant density of each DNA fraction was

determined based on the refractive index, measured using a Reichert AR200 digital refractometer (Depew, NY). The DNA fractions were extracted and recovered via ethanol precipitation. The recovered DNA was stored at -20 °C.

2.4.2 Detection of *pmoA* and *mmoX* Genes

Primer sets for *pmoA* and *mmoX* (Dedysh et al., 2008; McDonald et al.,2008; Rahman et al., 2011) were used to detect the presence of pMMO, sMMO and sMMO - *Methylocella* specific. For detecting the presence of pMMO, *pmoA* primer set, *pmoA* forward A189f (5'-GGNGACTGGGACTTCTGG-3') in combine with two different *pmoA* reverse primers mb661r (5'-CCGGMGCAACGTCYTTACC-3') and A692r (5'-GAASGCNGAGAAGAASGC-3'), were used. For sMMO (*mmoX)* forward mmoxf 945 (5'- TGGGGCGCAATCTGGAT-3') and reverse mmoxr 1401 (5'-TGGCACTCRTARCGGTC-3') were used. For detecting *Methylocella* genus-specific *mmo*X primer set, forward *mmoX*LF (5'- GAAGATTGGGGCGGCATCTG-3') and reverse *mmoX*LR (5'-CCCAATCATCGCTGAAGGAGT-3') were utilized. All primers were custom ordered from Integrated DNA Technologies, Inc. (Coralville, CA). Each 25-µL PCR mixture contained 400 nM forward/reverse primers set and 12.5 µL of Taq DNA polymerase PCR mix buffer (Qiagen, Valencia, CA). The PCR amplification reactions were conducted in an automated thermal cycler (PCR Sprint system, Thermo Electron Corp., Waltham, MA). The thermal cycling protocol is as follows: 95 °C for 15 min; 35 cycles of 95 °C for 1 min, 55 °C for 45 sec, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The presence of PCR amplicons was determined using gel electrophoresis.

2.5 Analysis of Active Microbial Community Structure

To characterize overall and active methane-oxidizing microbial community structure in each microcosm, real-time terminal restriction fragment length polymorphism (real-time-t-RFLP) assays were performed (Yu and Chu, 2005; Cho et al., 2013). Briefly, a region of 16S rDNA sequence (352 bp long) was amplified with a fluorescence-labelled forward primer 16S1055f (5C'-hexachlorofluorescein-ATGGCTGTCGTCAGCT-3C'), a reverse primer 16S1392r (5C'-ACGGGCGGTGTGTAC-3C'), and a *Taq*man probe 16S*Taq*1115f (5C'-[6-carboxyfluorescein]-CAACGAGCGCAACCC-[6-carboxytetramethylrhoda mine]-3C'). The copies in the samples were determined by comparing to standard curves with a range a known concentrations of plasmid no. 931. The plasmid no. 931 carries a partial 16S rRNA gene for *Nitrospira* (GenBank accession number AF420301). The PCR products were excised from a 0.9% agarose gel in 1% TAE buffer, recovered and purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA). The purified PCR products were then digested with restriction enzyme MspI (Promega Corp., Madison, WI), precipitated with ethanol, and resuspended in molecular-biology grade water. The lengths of T-RFs of digested PCR products were automatically determined on an ABI 3130xl Genetic Analyzer (DNA Technologies Core Lab, College Station, TX) by comparing internal standards (Mapmarker ROX-400 standards) using the Peak Scan 2.0 version. Genomic DNA extracted from microcosms receiving unlabelled methane were used as templates for determining overall microbial community structures. The ^{13}C-DNA fractions from ^{13}C-methane-amended microcosms were used as templates to determine the active methane-oxidizing microbial community structure. The real-time PCR reactions were performed using Bio-Rad iQ5 multicolor Real-Time PCR detection System (Hercules, CA). The results of terminal fragments were analyzed with online program MiCA3.

2.6 Cloning and Sequencing

The identities of active methane-oxidizing microorganisms were determined based on the 16S rRNA gene sequences derived from the ^{13}C-DNA fractions of the microcosms receiving ^{13}C-methane. The PCR reactions for 16S rRNA gene amplification were performed as described by Roh et al., 2009. Briefly, the amplified fragments of the 16S rRNA genes were cloned into the vector pCR4-TOPO using a TA Cloning Kit (One Shot TOP10 Competent cells) (Invitrogen, Carlsbad, CA). Colonies with inserts were verified by PCR with primers M13F (5´-GTAAAACGACGGCCAG-3´) and M13R (5´-CAGGAAACAGCTATGAC-3´), and screened on 0.9 % agarose gels (Fisher Scientific, Fair Lawn, NJ). For each ^{13}C-DNA fraction sample, seventy clones were randomly selected from the plates. The amplified 16S rRNA was purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA), followed by digestion with enzymes, HhaI and HaeIII (Promega Corp., Madison, WI). The clones with unique restriction fragment length polymorphism (RFLP) patterns on 4% metaphor agarose gels (Yu and Chu, 2005) were then selected for sequencing. The selected clones were grown overnight in 5 mL of Luria-Bertani (LB) broth with 50 mg/L kanamycin before use for plasmid extraction. The plasmids were extracted using a plasmid purification Kit (QIAGEN, Valencia, CA). The inserted sequences were determined by Eton Bioscience Inc. Phylogenetic analyses of aligned sequences were created using neighbor-joining approaching MEGA version 7.0.

2.7 Flow Through Aquifer Columns

A column study was conducted with site samples from the JBMDL Kirkwood formation to measure and assess the biodegradation of TCE under acidic, methanotrophic conditions, in a simulated model aquifer. Three columns were constructed from 16 gauge stainless steel tubing ~ 3.8 cm diameter x 25 cm length (see photographs in **Figure 10**). Both ends of the columns were sealed with Teflon plates (6.6 cm x 6.6 cm x 1.1 cm) topped with 1.1 cm polycarbonate plates for strength. The Teflon plates had a round 3.8 cm diameter x 0.55 cm deep channel cut into their face to accept a Viton Fluoroelastomer O-ring. This created a water tight seal with the stainless steel tubing used for each sediment column. The columns were assembled using 4 threaded rods which passed through the square Teflon and polycarbonate plates at each corner. The rods were attached to the plates using washers and nuts. As the columns were operated in an up-flow mode, a Teflon diffuser was placed at the bottom of each column to reduce channeling and supply an even water flow throughout the column. The diffuser was made from a 0.14 cm-thick Teflon sheet perforated with 0.14 cm holes. A 100 mesh stainless steel screen was placed over the diffuser and at the top of the column soil to contain fine soil particles.

The three columns were initially packed with the Kirkwood formation aquifer solids, using a metal rod to compact the materials as the columns were filled (compacting after each ~ 50 g added). The wet weights of the aquifer solids were 446, 442, and 426 g for column 1 (C1), column 2 (C2) and column 3 (C3), respectively. Based on a 23 % measured soil moisture, this equates to 343, 340, and 328 g aquifer solid dry wt for C1, C2, and C3, respectively. After each column was full, the top plates were added and the system was secured with threaded rods, washers and nuts.

Groundwater was collected into a ~ 60 L sterilized stainless steel keg from well M204 at the McGuire AFB Mag 1 plume. This keg was used as a source of water for each of the columns. The water was initially pumped into each of two Flex-foil bags. One bag (5 L) received 4L of water, 100 mL of pure oxygen, and 50 mL of pure methane gas. This bag was used as the reservoir for C1 and C2, which were the columns fed methane. A second 3-L Flexfoil bag received 2L of site groundwater and 50 mL of oxygen (no methane). This bag served as the water feed for C3, the aerobic control without methane. The bags were mixed and then each was hooked to the influent end of one column and the water was pumped to the bottom of the columns through Teflon-lined Tygon tubing (0.28 -cm inner diameter, 0.14-cm outer diameter) using a peristaltic pump with Norprene tubing in the pump-head. TCE was not initially added. Over the course of the study, the Flex-foil bags were refilled with groundwater and gases added as necessary, with adjustments made depending on the column conditions, as described in the *Results and Discussion* section. The columns were operated in a cold room set to maintain at 15 – 17 ^{o}C.

Groundwater flow was initially set to 3.8 mL/hour (~ 24 hr hydraulic residence time; HRT). For 86 days, columns were run under the conditions, described above with the goal of increasing the methanotroph population in the aquifer materials prior to the addition of TCE. Dissolved oxygen, methane, and pH values were measured twice weekly in both the column influent and effluent. On Day 87, TCE was added to the column influent using syringe pumps, and was thereafter added to the column sampling and analytical regime. The target influent TCE concentration was ~ 100 μg/L. On Day 138, C1 and C2 were connected to increase the total residence time in the methane-amended treatment to ~ 48 hrs. Copper was added to the influent groundwater on Day 163 and thereafter in an attempt to stimulate methanotrophs with pMMO. The column study was terminated after ~ 212 days of operation.

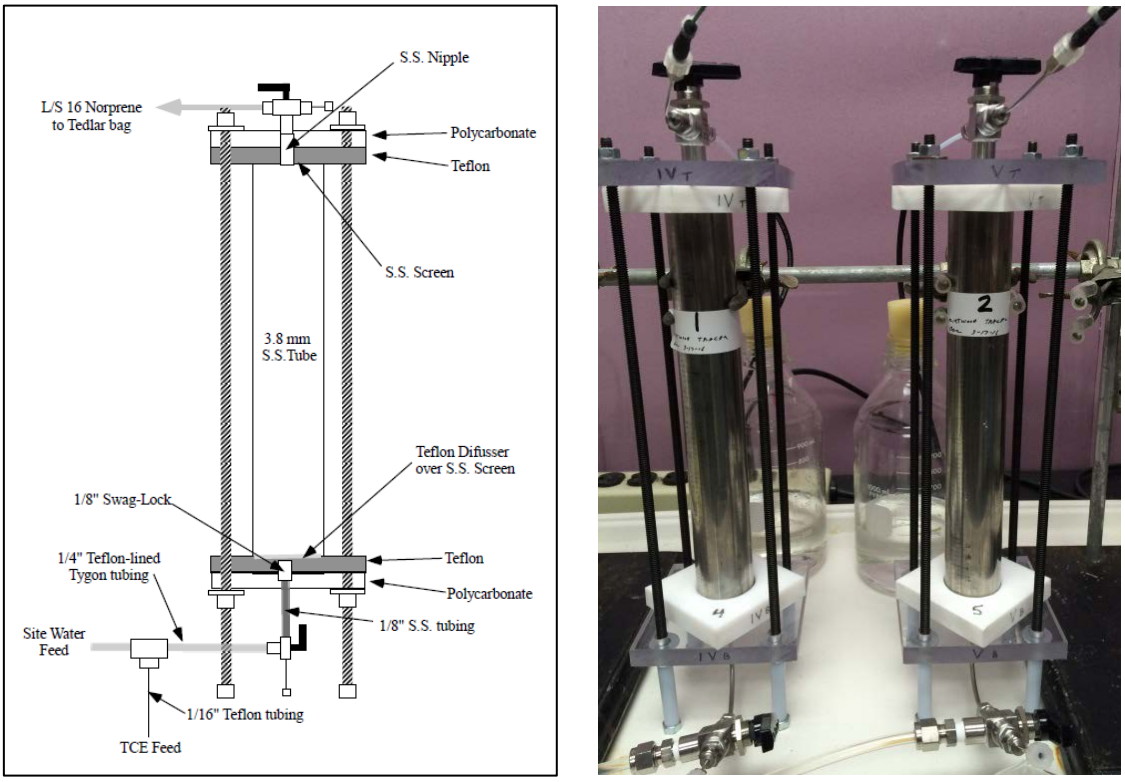


Figure 10. Diagram and photograph of columns used in flow-through aquifer study.

3.0 RESULTS AND DISCUSSION

3.1 Microcosm Studies

3.1.1 Joint Base McGuire-Dix-Lakehurst (JBMDL)

Kirkwood Formation. The initial pH in the Kirkwood microcosms was ~ 3.4. The pH in the microcosms rose somewhat over the course of this study, but was less than 4.0 in all bottles at the conclusion of the study (**Figure 11**).

The consumption of methane in the sample bottles was observed after the first week of incubation, with calculated aqueous concentrations declining from ~ 1,650 µg/L on Day 7 to < 1 µg/L on Day 19 (**Figure 12**). Methane was consumed irrespective of the presence or absence of TCE. Headspace methane was re-added on Day 20, and three additional times after it was observed to have been depleted (days 20, 25, 33, and 36). In contrast, there was little (~12%) methane loss in the killed controls over the course of this study. On days 33 and 36, half of the bottles (i.e., 3) in each treatment received ^{13}C-labeled methane, while the other half continued to receive ^{12}C-methane. On Day 35 and again at the conclusion of the study (19 or 21 days after initial $^{13}CH_4$ addition for samples), slurry samples were taken from the microcosms, preserved by freezing at -20 ^{o}C, and microbial community analysis was performed via SIP.

There was a lag period of ~ 3 weeks before TCE degradation was detectable in the bottles (**Figure 13**). TCE was reduced from 392 ± 40 µg/L to 74 ± 40 µg/L over the following 5 weeks. A first order rate constant of 0.050 day^{-1} was calculated for TCE (r^2 = 0.96) from Day 21 to Day 55. TCE in the killed controls also declined somewhat over time, being reduced from 339 ± 71 µg/L to 267 ± 54 µg/L over 8 weeks of incubation. The reduction in TCE in the killed controls was likely due to volatile losses, although another type of abiotic loss also cannot be ruled out. The first order rate constant for the loss of TCE in the killed samples from Day 21 to Day 55 was 0.019 day^{-1} (r^2 = 0.95), or about 2.5 times lower than for the live samples. These data clearly indicate the biodegradation of both methane and TCE in microcosms with a starting pH of only 3.4. The data are consistent with those from the MBAFB site, where methane degradation was observed at pH < 4 (**Figure 5**) and enrichments from the site degraded TCE, *cis*-DCE and VC (**Figure 6**). cVOCs were not present in the original microcosms from MBAFB as this study was conducted to evaluate 1,4-dioxane degradation.

Vincentown Formation. Aquifer solids were also collected from the Vincentown formation, which underlies the Kirkwood formation, and is readily distinguished by the dark greenish color of its glauconitic sands (Tedrow, 2002). Galuconite is a hydrous silicate of ferrous and ferric iron, magnesium and alumina, and was once widely utilized as an agricultural additive, in part because some deposits contain up to 8% potash (K_2O) (Tedrow, 2002). The dissolved oxygen in the MAG204 well was < 0.2 mg/L, although the ORP was slightly oxidizing at +131 mV. The pH was ~4.4 in the microcosms (data not shown). Oxygen consumption was observed in the live and killed microcosms over the course of 6 weeks, although the extent of consumption was generally greater in the live samples (**Figure 14**). It is likely that abiotic oxidation of Fe (II) or other reduced minerals accounted for the O_2 consumption in killed samples. No significant losses of methane were observed in the live or killed samples over 5 weeks, despite the continuing oxygen consumption (**Figure 15**). Based on the data, this study was discontinued after 6 weeks, and the focus of SIP and enrichments was placed on the Kirkwood formation

samples. It is possible that the dominant oxidation of Fe and other minerals in these samples was preventing methane oxidation, at least over the course of the short study, and that this process may have occurred over a longer timeframe. However, given that this is a rather unusual aquifer formation (i.e., glauconite) that does not represent conditions in a majority of coastal plain aquifers, the study was discontinued.

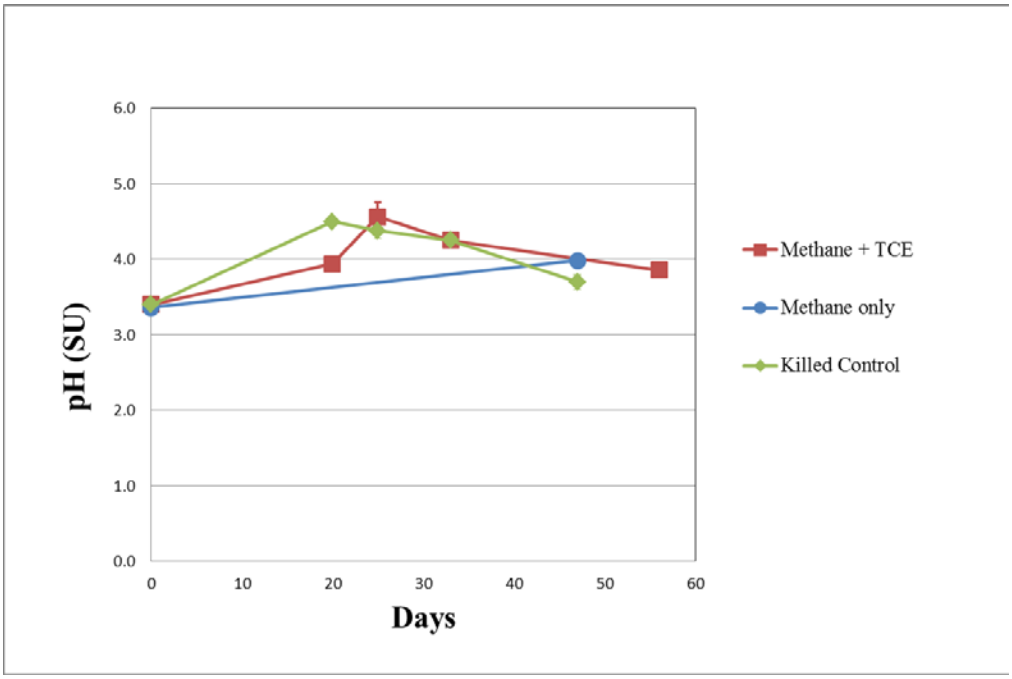


Figure 11. pH in JBMDL Kirkwood formation microcosms. Error bars represent the standard deviations from 4-6 replicates.

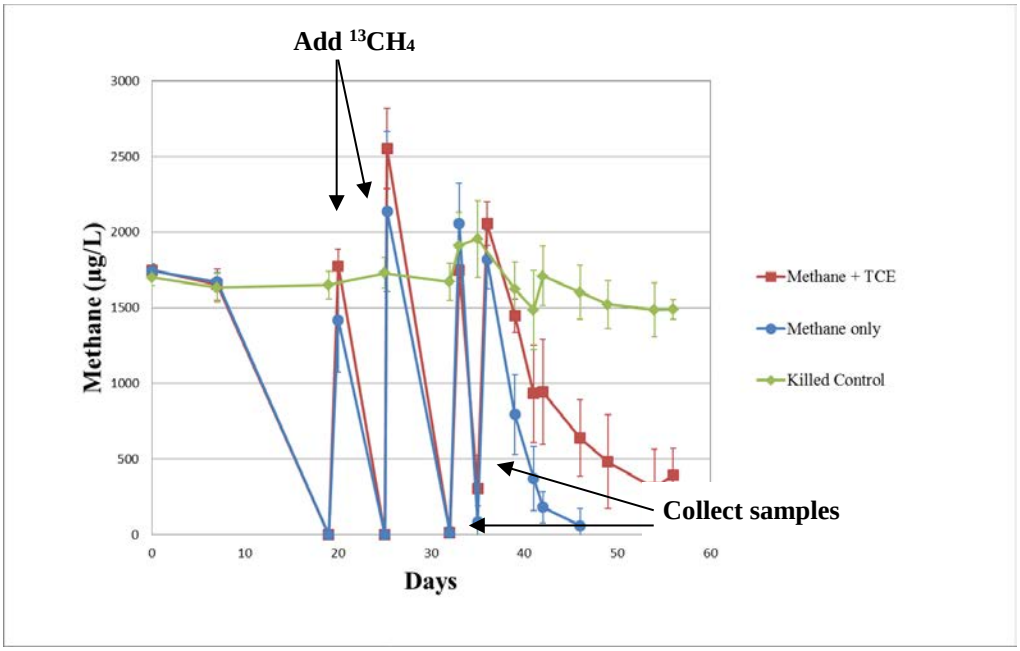


Figure 12. Methane consumption in JBMDL Kirkwood formation microcosms. Error bars represent the standard deviations from 4-6 replicates, and arrows denote where $^{13}CH_4$ was added and subsamples were collected (19 and 21 days after the second addition).

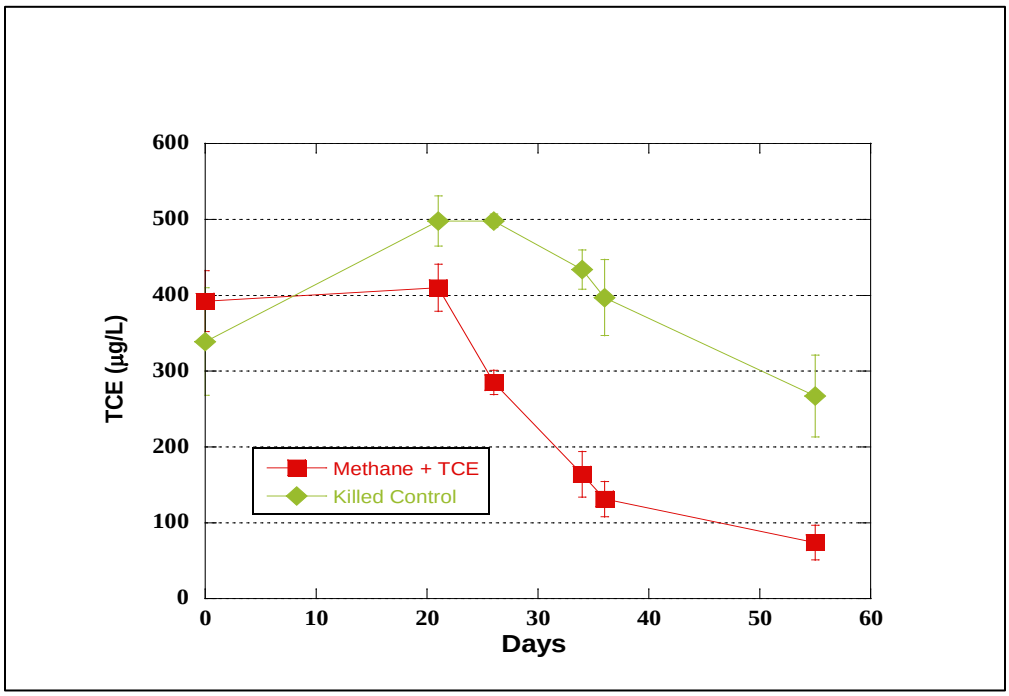


Figure 13. TCE concentrations in JBMDL Kirkwood formation microcosms. Error bars represent the standard deviations from 4-6 replicates.

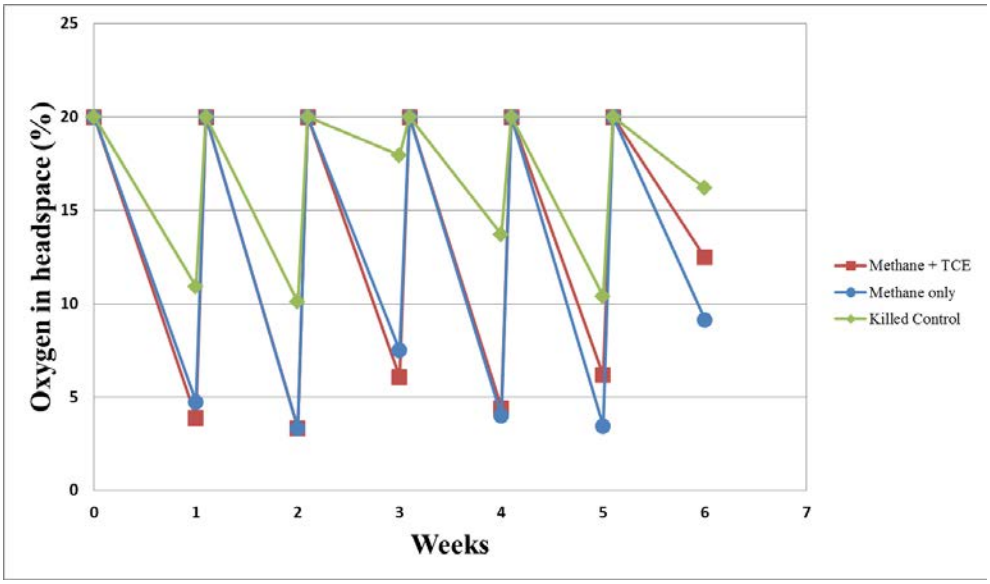


Figure 14. Oxygen levels in Vincentown formation microcosms.

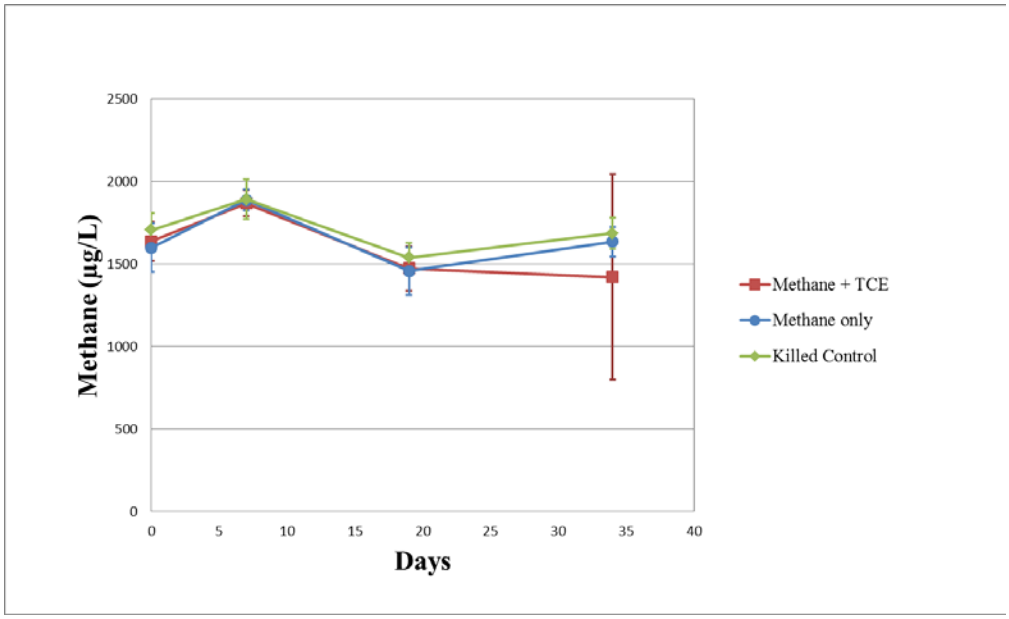


Figure 15. Methane concentrations in JBMDL Vincentown formation microcosms. Error bars represent the standard deviations from 6 replicates.

3.1.2 Naval Surface Warfare Center Dahlgren, VA (NSWC Dahlgren)

Methane was completely consumed within 2 weeks in treatments receiving 1% and 10% methane with nutrients (**Figure 16**). In treatments receiving 0.1% methane, consumption took significantly longer (i.e., 4 weeks or more). This disparity in methane consumption rates is likely due to the fact that biomass was not generated as quickly at the lower methane level. There was no significant consumption of ethane or ethene in the Dahlgren microcosms (data not shown). The pH remained between pH 4.0 and 5.0 in all microcosms for the duration of this study (69 days; **Figure 17**).

TCE concentrations in the initial set of NSWC Dahlgren microcosms that received methane and nutrients, as well as live and killed controls, are presented in **Figure 18**. There was a steady decline in TCE in all treatments, including the killed controls. The reason for this is unclear, but may be due to sorption to microcosm solids, volatile losses, or a combination of these factors. The reduction in TCE in the microcosms receiving 10% methane in the headspace was significantly greater than that in the killed controls or other samples. In the 10% methane treatment, TCE declined from 201 ± 3 µg/L to 55 ± 9 µg/L over the 10 week study. The first order rate constant, including data from Day 14 to Day 69, excluding the initial apparent lag phase, was 0.02 day^{-1} (R^2 = 0.89). By comparison, TCE in the killed samples declined from 214 ± 6 µg/L to 121 ± 29 µg/L over the 10 week study, and the live microcosms with nutrients, but no methane added declined only from 179 ± 2 µg/L to 146 ± 11 µg/L, over the same timeframe. The first order rate constants for these treatments, calculated over the same period as the 10% methane treatment were 0.009 day^{-1} (R^2 = 0.90) and 0.004 day^{-1} (R^2 = 0.43), respectively. Thus, samples with 10% methane showed the highest decline in TCE, and a mean rate constant that was 2x to 5x that of other relevant treatments without methane or those with lower concentrations of methane added. The high methane concentration used in these microcosms likely yielded higher numbers of methanotrophs over the short course of the study, thus leading to measurable TCE degradation over the background of volatile/sorptive losses. Based on these results, a second Dahlgren microcosm study was prepared specifically for the purpose of SIP analysis.

The values pH values in some of the replicate microcosms set-up for SIP analysis from the NSWC Dahlgren site were ~2 rather than ~4.5. The reason for the low pH in these microcosms is unclear. No methane consumption occurred in any of the microcosms at pH 2, so they were not used further in the SIP study. Four of the 6 microcosms that received nutrients, 10% methane and TCE had a pH of ~ 4.5, and these were selected for the SIP analysis along with killed controls. Methane consumption in these microcosms at pH ~4.5 is presented in **Figure 19**. Methane degradation proceeded at a slightly slower initial rate in these microcosms than in the initial study, with methane consumption nearly complete after 3 weeks of incubation, compared with 2 weeks in the initial study. ^{12}C-methane was re-added on Day 54; this methane was completely consumed within 8 days. At day 81, microcosms received either ^{12}C- or ^{13}C-methane; this methane was then consumed within 3 days. The increasing rate of methane consumption is consistent with increasing methanotroph biomass in the microcosms. Samples for SIP and microbial community analysis were collected from the microcosms on Day 82, 83 and 84 (**Figure 20**).

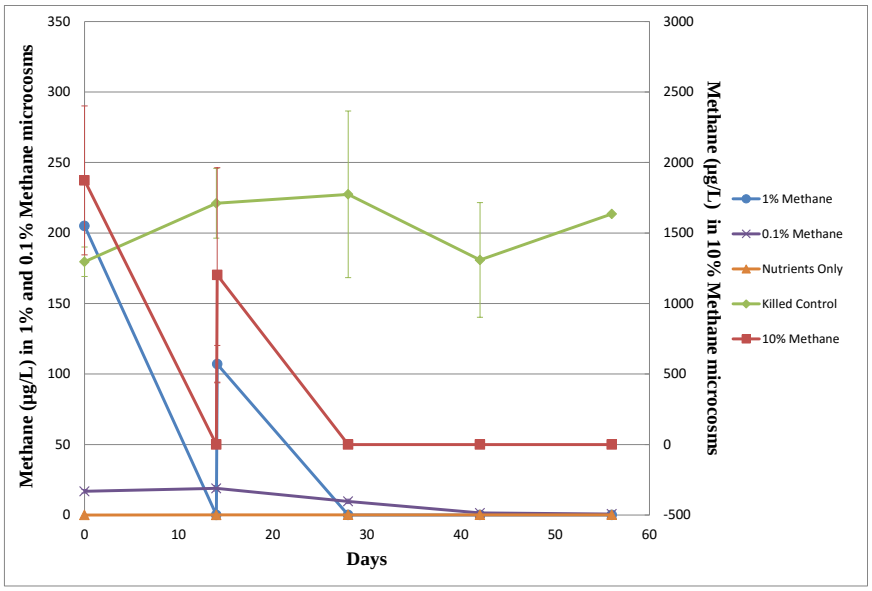


Figure 16. Methane concentrations in NSWC Dahlgren microcosms. Values are means and standard deviations from duplicates.

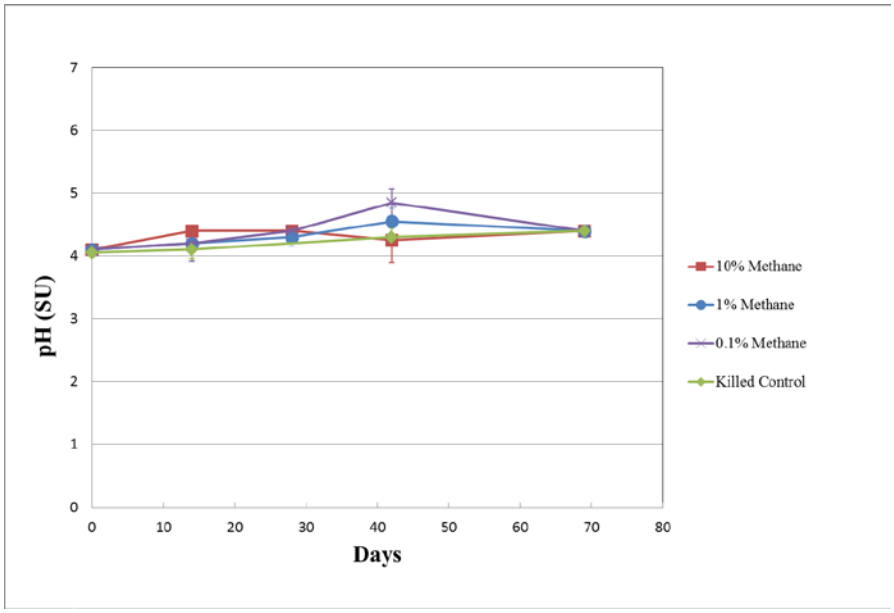


Figure 17. pH in NSWC Dahlgren microcosms. Values are means and standard deviations from duplicates.

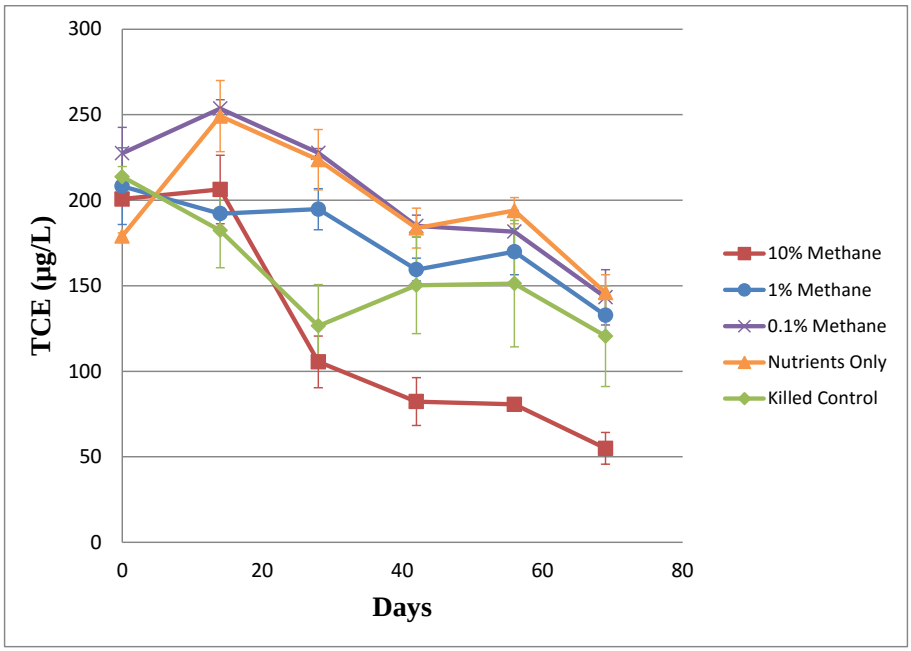


Figure 18. TCE concentrations in NSWC Dahlgren microcosms. Values are means and standard deviations from duplicates.

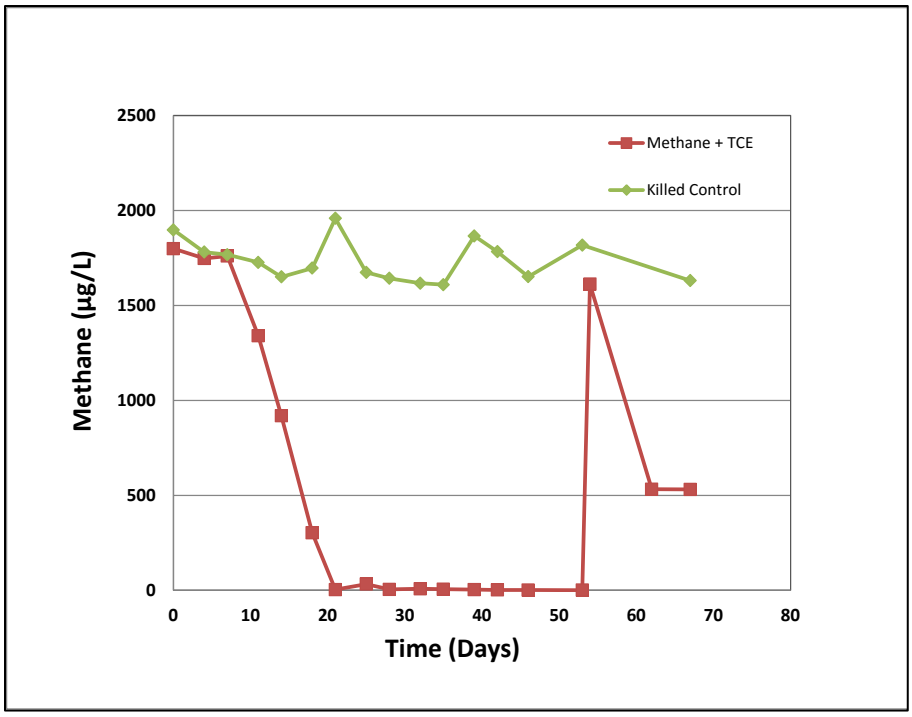


Figure 19. Methane concentrations in NSWC Dahlgren microcosms used for SIP analysis over the first 70 days.

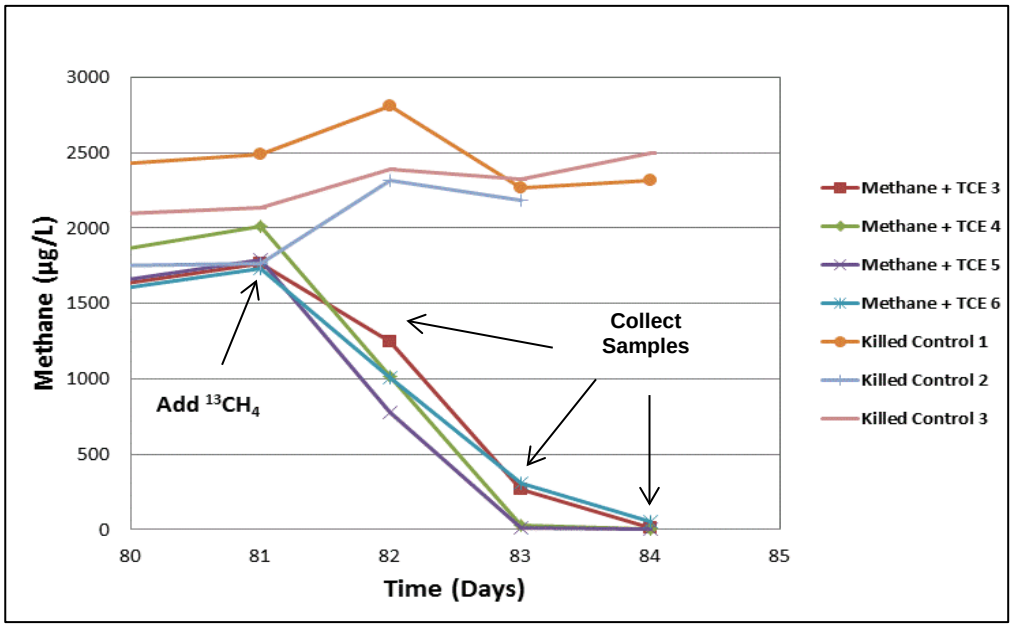


Figure 20. Methane concentrations in NSWC Dahlgren microcosms used for SIP analysis after addition of ^{13}C-methane.

3.1.3 Indian Head Division, Naval Surface Warfare Center, MD (IHDIV)

The groundwater used in the IHDIV microcosms had a measured pH of 4.7. Once microcosm set up was complete, the pH of the microcosms ranged from 5.0 to 5.3, presumably due to soil buffering capacity (**Figure 21**). After 18 days of incubation, the pH in the microcosms was pH 5.7-5.8, which is above the desired range for this study. The pH was adjusted to pH ≤5 using HCl at this time. At 47 days of incubation, the pH was again found to range from 5.5-6.0. The pH again was adjusted to ≤5 using HCl. Following this adjustment, the pH in the microcosms remained at or below pH 5.3 for the remainder of the study. The pH slowly increased in the killed controls as well as the live samples, suggesting that it was abiotic reaction(s) rather than a biological reaction that caused the pH increase in the bottles.

Headspace oxygen was brought to 20% at the beginning of the study, and oxygen was typically added whenever the headspace concentrations dropped below 15% (**Figure 22**). There was significant oxygen consumption in all samples over the first day of incubation, with headspace concentrations dropping to 10% or less in all treatments, including the killed control. This likely reflects abiotic oxidation of minerals or other reduced elements in the samples. Over time, the oxygen consumption in the killed samples was appreciably reduced, while that in the samples with 10% methane or ethene continued, with rates of oxygen consumption in the ethene bottles appearing to increase over time (**Figure 22**). This clearly represents oxygen consumption tied to the biological oxidation of methane or ethene.

Consistent with the oxygen data, methane was rapidly and repeatedly consumed in the IHDIV microcosms. All of the original methane was consumed within two weeks of incubation. Methane was re-added on three occasions, and after each addition, the methane was consumed within 1-2 weeks (**Figure**

22). Ethene was also consumed in IHDIV microcosms (**Figure 23**). The original 10% in headspace (resulting in a calculated 12,000 µg/L in the water phase) was degraded in ~ 40 days. The respike on Day 54 was completely consumed in ~ 20 days. Clearly organisms capable of degrading methane and ethene were present in the site samples, whose pH was 4.7 when groundwater was collected.

The groundwater used in this study contained ~ 25 µg/L of TCE and 6 µg/L of cis-DCE upon sampling in March, 2016 (baseline data for another study). The dissolved oxygen was just under 2 mg/L and the ORP was + 225 mV. The well also had a trace of methane (6 µg/L), and relatively low concentrations of both nitrate (0.8 mg/l) and sulfate (3.4 mg/L). TCE was added to the microcosms to ~ 300 µg/L. Interestingly, there was a significant decline in TCE in all of the sample treatments, including the killed controls, over the first 18 days of incubation (**Figure 25**). The overall extent of TCE loss was significantly less in the killed controls than in the live treatments, with TCE declining to 134 ± 2 µg/L in the killed control and between 43 and 77 µg/L in the various live treatments over 89 days. There was also production of *cis*-DCE in many of the same treatments over the first 18 days, with the most significant increases occurring in the killed controls, and the least in samples with 10% methane (**Figure 26).** The combined data seem to indicate than an initial reductive process occurred in the samples, resulting in some production of *cis*-DCE. A trace of VC was also observed in the killed controls and samples with ethene (**Figure 27**). It is possible that this was an abiotic process (based upon the killed control results), although *trans*-DCE would also be expected in that case, which was not detected, or that the killed controls possessed some biological activity, resulting in some initial reductive dechlorinating of TCE.

Interestingly, the *cis*-DCE in the killed control samples increased with time over the 89-day study, reaching a maximum of ~ 80 µg/L (**Figure 26**). In the bottles with ethene, however, the *cis*-DCE was at a maximum concentration after 18-days, and then sharply decreased, being below detection (< 2.6 µg/L) by Day 56. In bottles with 1% methane or no methane added, the *cis*-DCE that was formed over the first 18 days persisted over the course of the study, whereas in the samples with 10% methane, the *cis*-DCE reached a lower maximum concentration after 18 days (~ 15 µg/L) than other treatments, and slowly declined to 5.6 µg/L over 80 days. One interpretation of these data is that the initial formation of *cis*-DCE by a reductive process was subsequently followed by its aerobic oxidation in samples with ethene and methane, at least when present at high enough concentrations to support significant methanotrophic activity (i.e., 10%). Ethene-oxidizing bacteria are known to oxidize *cis*-DCE and VC (e.g., Mattes et al., 2005, 2010) which was also formed at low concentrations in the bottles with ethene, and subsequently degraded (**Figure 27**). However, the pH tolerance of these organisms and/or their occurrence in acidic groundwater aquifers has not been studied to our knowledge. Overall, while the initial apparent reduction of TCE was unexpected, this study seems to illustrate that oxidative destruction of the common daughter products *cis*-DCE and VC is feasible at the pH of these samples.

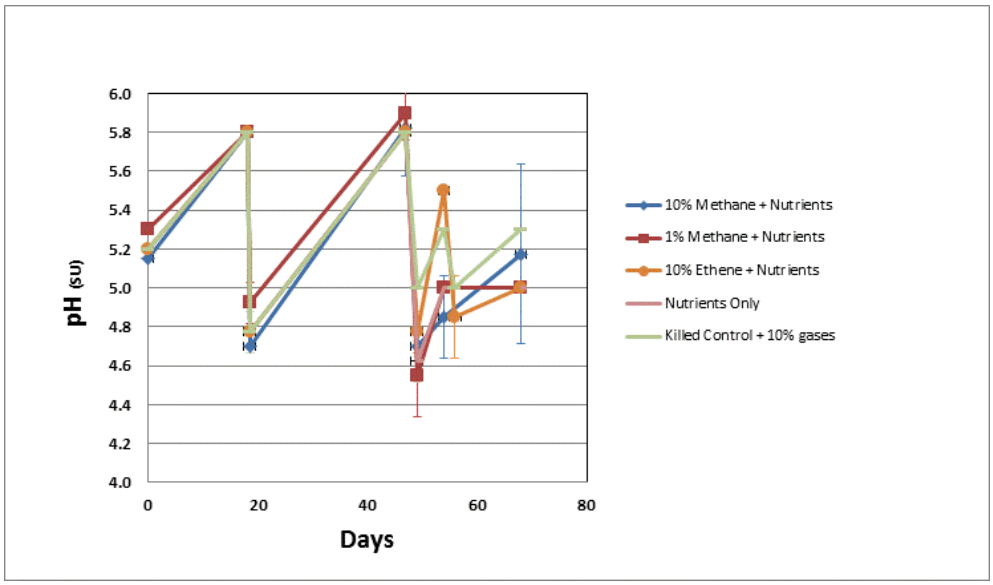


Figure 21. pH in IHDIV microcosms. HCl was added on Days 18 and 47 to reduce pH below 5.

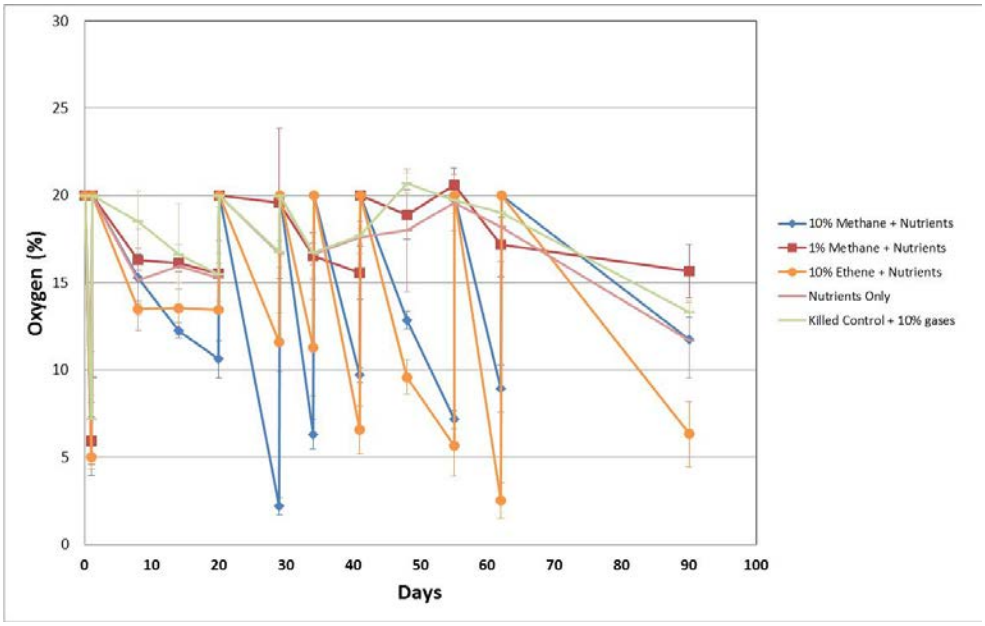


Figure 22. Dissolved oxygen in IHDIV microcosms. Oxygen was generally brought back to 20% in headspace when concentrations < 15% were detected.

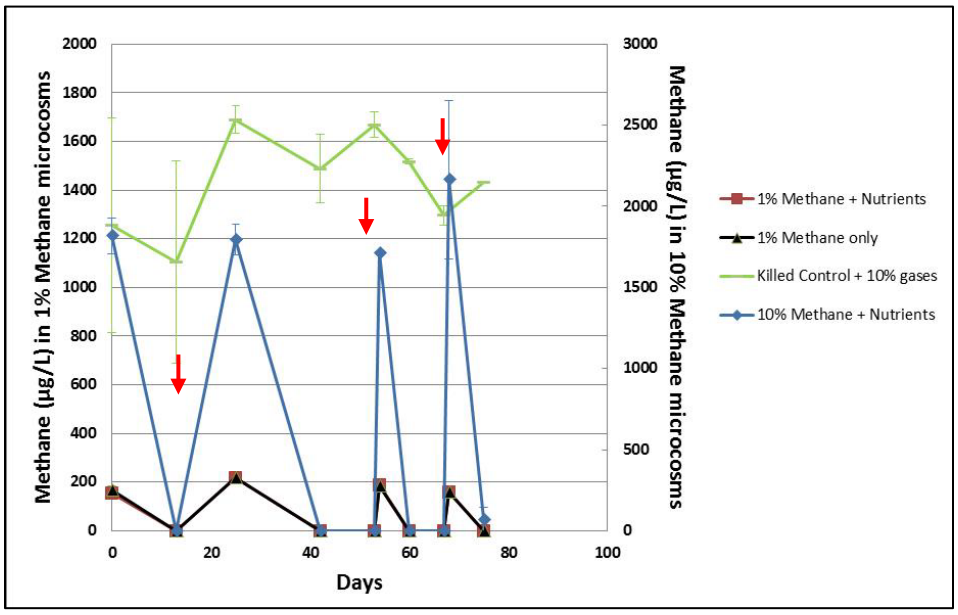


Figure 23. Methane concentrations in IHDIV microcosms. Additional methane was added at the times indicated with a red arrow.

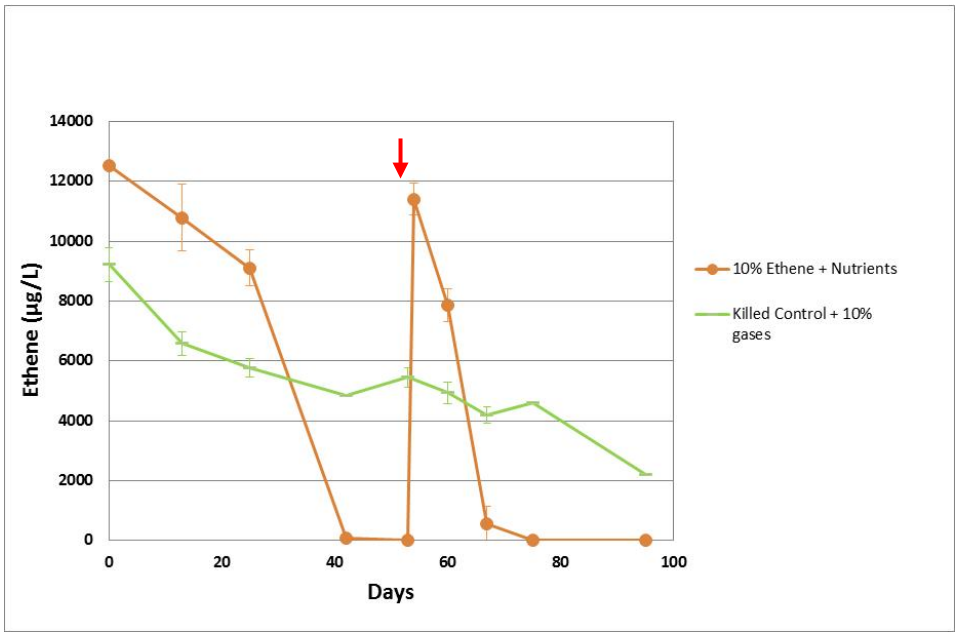


Figure 24. Ethene concentrations in IHDIV microcosms. Additional ethene was added at the time indicated with a red arrow.

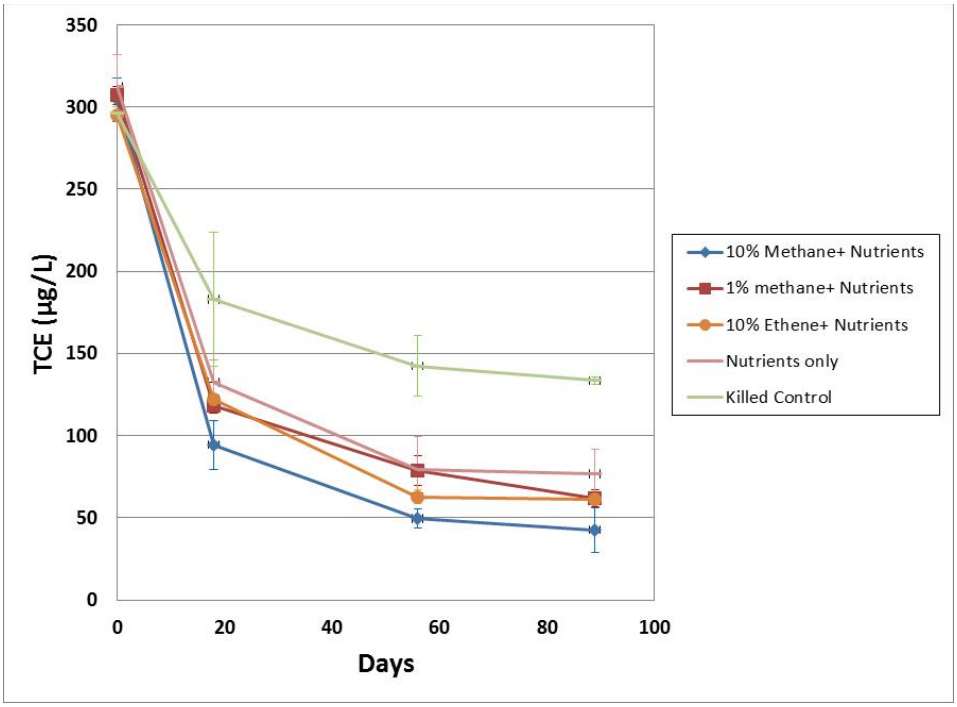


Figure 25. TCE concentrations in IHDIV microcosms. Error bars are standard deviations from duplicate samples.

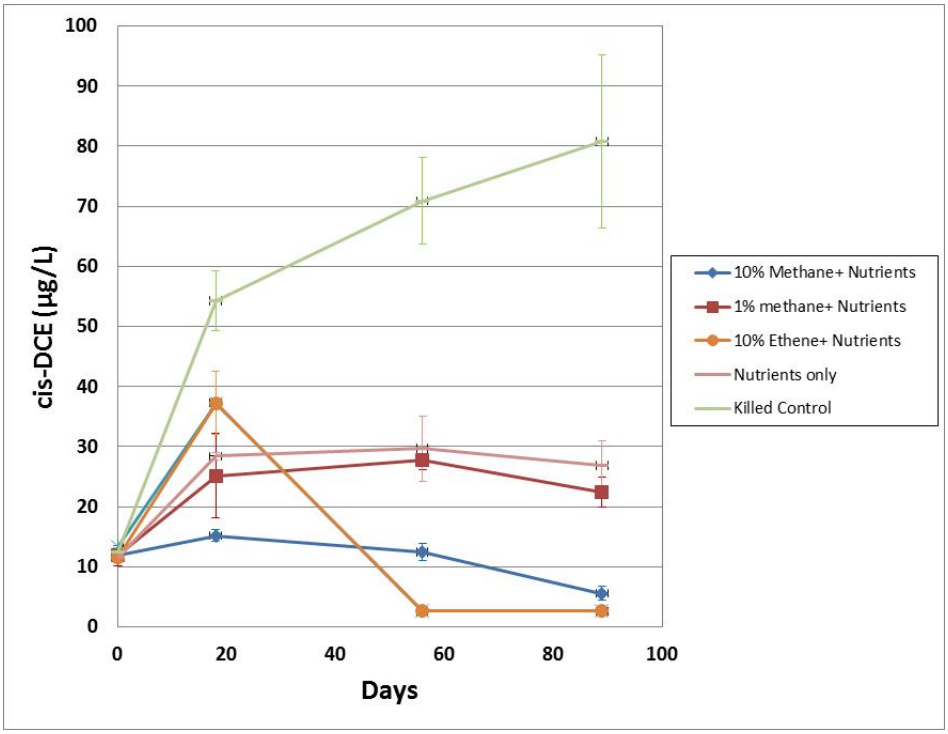


Figure 26. *Cis*-DCE concentrations in IHDIV microcosms. Error bars are standard deviations from duplicate samples.

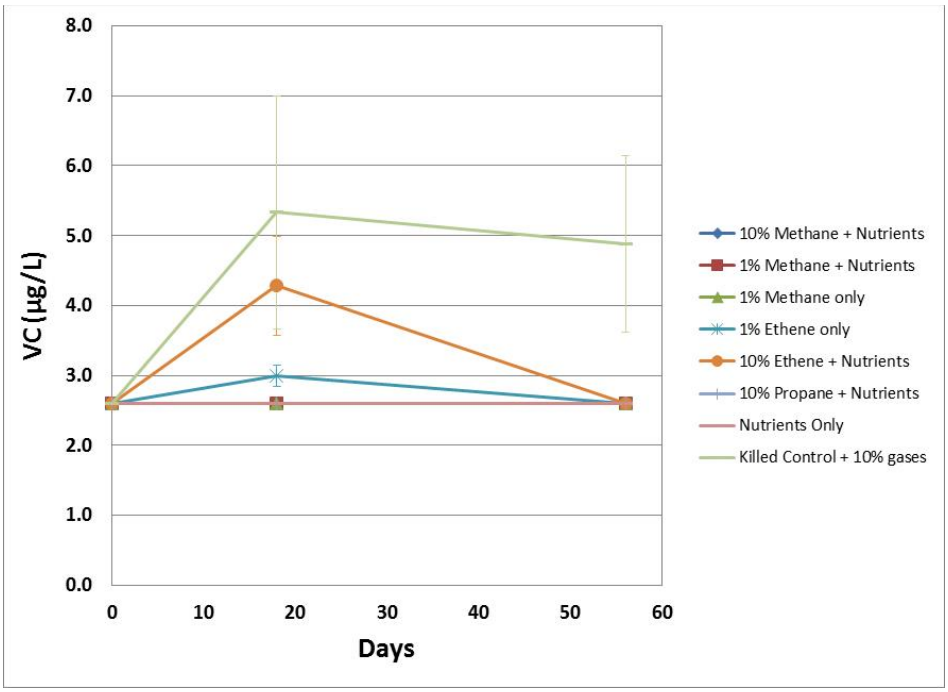


Figure 27. VC concentrations in IHDIV microcosms. Error bars are standard deviations from duplicate samples.

3.2 Microbial Enrichments

Microbial enrichments on methane (and ethane or ethene for some samples) were initiated using site soils from the three locations described previously, and the ability of these enrichment cultures to biodegrade various cVOCs at acidic pH was examined. A photo showing enrichment cultures is provided in **Figure 29**.

3.2.1 JBMDL Enrichments

For the JBMDL site, enrichment cultures were initiated with methane, ethane, and ethene. The most significant growth was observed in a culture with ethane at pH 4.0. This culture was transferred three times and then evaluated for degradation of TCE, *cis*-DCE and VC. Cultures were not obtained from this site with methane or ethene as substrates.

The ethane culture was observed to biodegrade TCE, *cis*-DCE and VC in ATCC 2157 medium at pH 4, with an initial concentration of 3% ethane added to the headspace after the culture was washed (**Figure 28**). These data suggest that ethane, in addition to methane, could be an important substrate for enhancing degradation of cVOCs in acidic aquifers. Ethane, even though it occurs as a degradation product of various cVOCs, has received relatively little study as a cometabolic substrate (e.g., Freedman and Herz, 1996; Verce and Freedman, 2000; Hatzinger et al., 2015), and there has been no specific focus on its potential contribution to cVOC degradation in low pH environments. Because the rates were fairly slow, the TCE study was repeated a second time, and degradation was confirmed (data not shown).

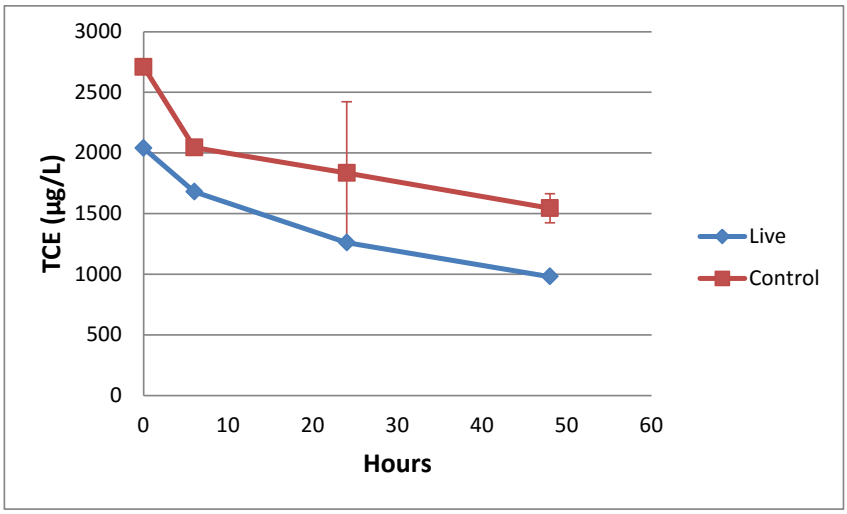
3.2.2 NSWC Dahlgren Enrichments

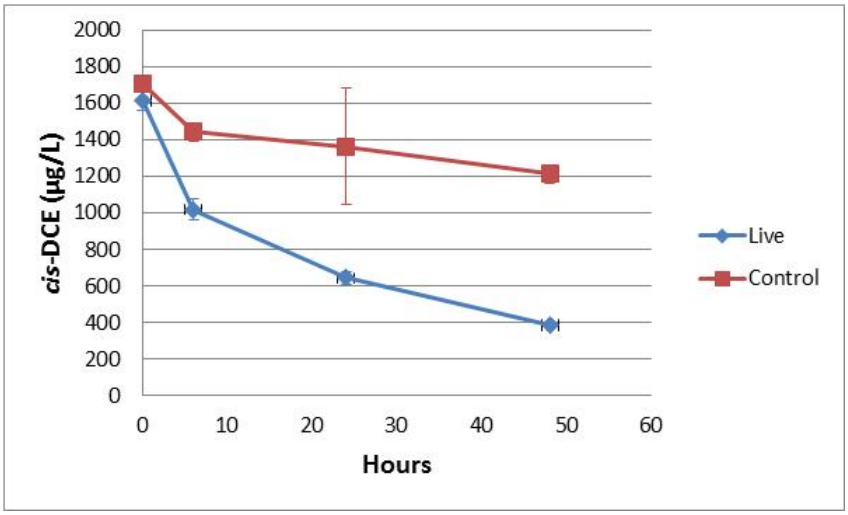
For the NSWC Dahlgren site, enrichment cultures also were initiated with methane, ethane, and ethene. Cell growth was observed in both the methane and ethane cultures at pH 4.0, but not in enrichment cultures with ethene. In an attempt to selectively grow methanotrophs possessing pMMO, Cu was added to some of the enrichment media to specifically stimulate this enzyme. Other enrichments were Cu free to selectively stimulate organisms with sMMO. After multiple transfers, the cultures were evaluated for degradation of various cVOCs, including TCE, *cis*-DCE and VC.

A methane-enrichment culture grown at pH 4.0 with Cu in the media and formate as a source or reducing equivalents was observed to biodegrade TCE and VC, but not *cis*-DCE **(Figure 30)**. This is an interesting observation because previous studies with pMMO-containing methanotrophs suggest that *cis*-DCE is a substrate for this enzyme, although degradation rates may be somewhat slower than for sMMO (Anderson and McCarty, 1997; Semrau, 2011). Thus, the results herein suggest that the specificity of degradation of cVOCs may differ under low pH conditions, and for cultures that thrive under these conditions, than for typical methanotrophs that grow optimally at neutral pH. This culture also degraded 1,1,1,-trichloroethane (data not shown).

A methane-degrading culture from the Dahlgren site was also tested for cVOC degradation under Cu-limiting conditions in order to assess the potential for sMMO induced degradation of cVOCs at low pH. This culture was tested to evaluate degradation of several cVOCs, including PCE, TCE, *cis*-DCE, VC, 1,1,-dichloroethene (1,1-DCE), 1,1-dichloroethene (1,1-DCA), 1,2,-dichloroethane (1,2-DCA) 1,1,1-TCA, 1,2-dichloromethane (DCM), and chloroform (CF). In multiple tests, the culture was observed to biodegrade *cis*-DCE, 1,2-DCA, and DCM (data not shown). Data for other compounds, including TCE 1,1,-DCA, 1,1-DCE, and CT were inconclusive, showing some level of loss, but not enough to confirm degradation given the variability of the assay. No degradation was apparent in multiple trials for PCE, 1,1,1-TCA, or 1,1-DCE.

Figure 28. Photograph of various cometabolic enrichment cultures. The filters on each Erlenmeyer flask are used to add gases to the bottles without introducing bacteria.





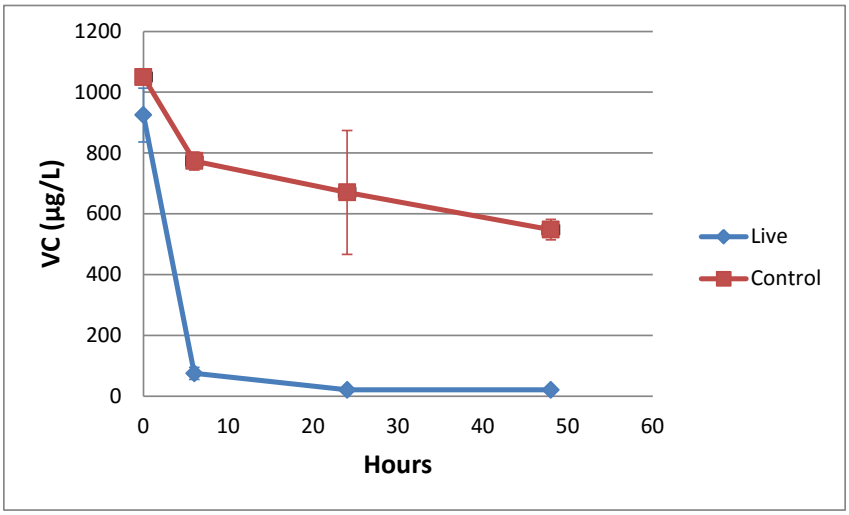


Figure 29. Biodegradation of TCE (top panel), cis-DCE (middle panel) and VC (bottom panel) at pH 4 by the JBMDL enrichment culture grown on ethane. Error bars are standard deviations from duplicate samples.

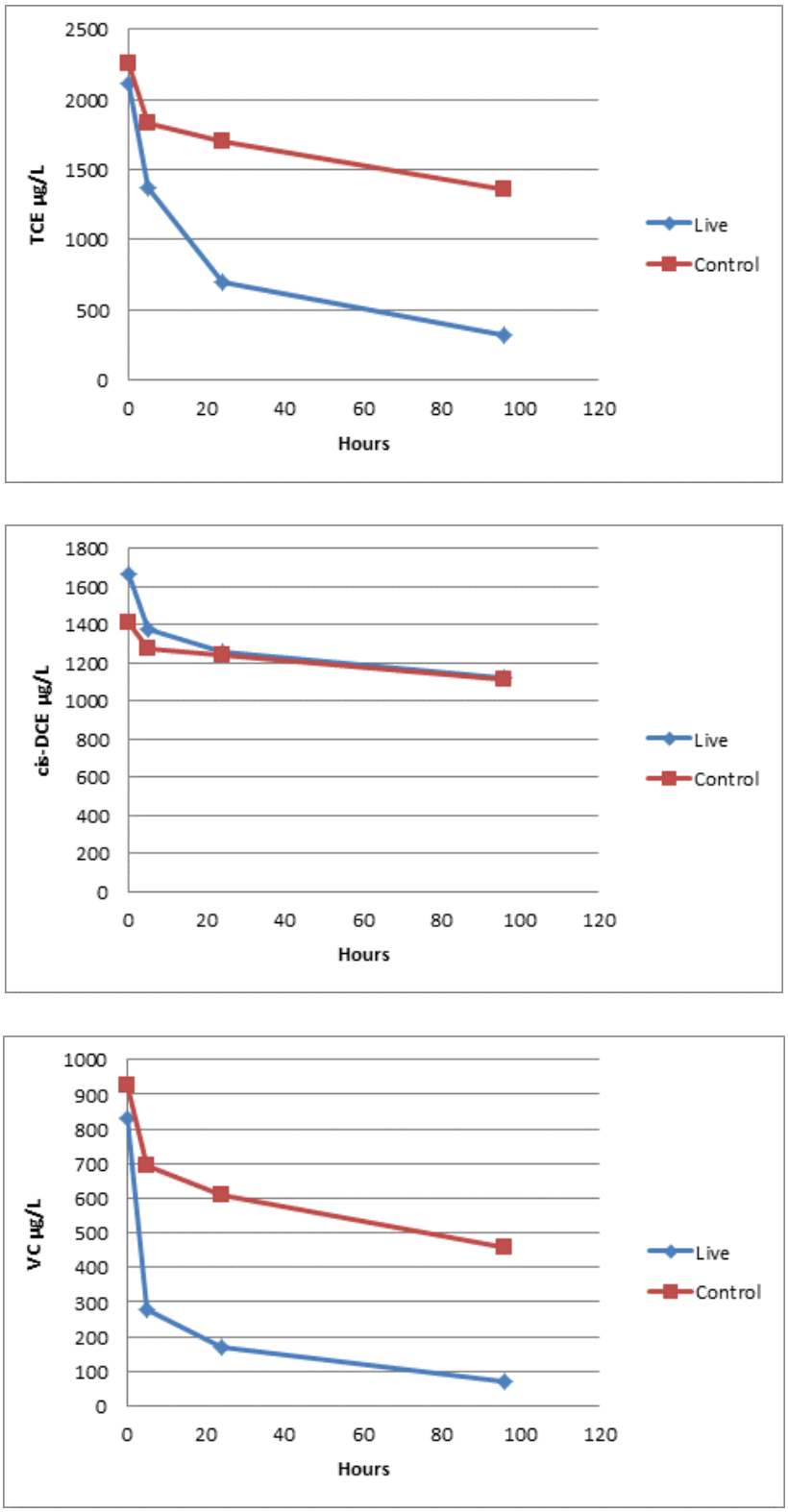


Figure 30. Biodegradation of TCE (top panel), *cis*-DCE (middle panel) and VC (bottom panel) at pH 4 by a NSWC Dahlgren enrichment culture grown on methane with Cu. Error bars are standard deviations from duplicate samples.

3.2.3 IHDIV Enrichments

As described for the other two sites, for the IHDIV site, enrichment cultures were initiated with methane, ethane, and ethene. In this case, cell growth was observed in both the methane and ethene cultures at pH 4.0, but not in enrichment cultures with ethane.

An enrichment culture grown on ethene was observed to rapidly biodegrade VC and *cis*-DCE, but not TCE at pH 4 (**Figure 31**). The *cis*-DCE and VC data are consistent with the microcosm study from this site in which both of these cVOCs were biodegraded in bottles that received ethene gas. Previous studies have shown that ethene can support VC and cis-DCE oxidation (Mattes et al., 2005, 2010), but to our knowledge, this is the first report of this activity at a pH as low as 4.

An enrichment culture (IH57) was also established from this site with methane as a sole carbon source. This culture was grown in media with and without Cu on multiple occasions and then tested for degradation of various cVOCs. In the presence of Cu, the culture was observed to biodegrade TCE and VC, but not to degrade *cis*-DCE (or to degrade it very slowly) in multiple trials in the presence or absence of formate. Data from one study without formate are provided in **Figure 32**. Interestingly, these data are very similar to the results observed for the enrichment culture from the Dahlgren site cultivated under similar conditions. Further study is warranted to better understand the selectivity of the presumptive pMMO that is catalyzing reactions under these conditions.

In the absence of Cu, the results from multiple trials were somewhat less consistent than for the treatments with Cu. In an initial study with the enrichment culture (IH57) in Cu-free media, clear degradation of TCE, *cis*-DCE, VC, 1,2-DCA, CF, and DCM were observed (**Figure 33**). In two later experiments, the results were less clear, but this may reflect induction of the sMMO enzyme, absence of reducing equivalents (e.g., formate, which was not added in these studies) or possibly changes in the IH57 culture over time. Further evaluation was not possible in this limited scope study, but the initial results clearly demonstrated degradation of several cVOCs by this enrichment culture.

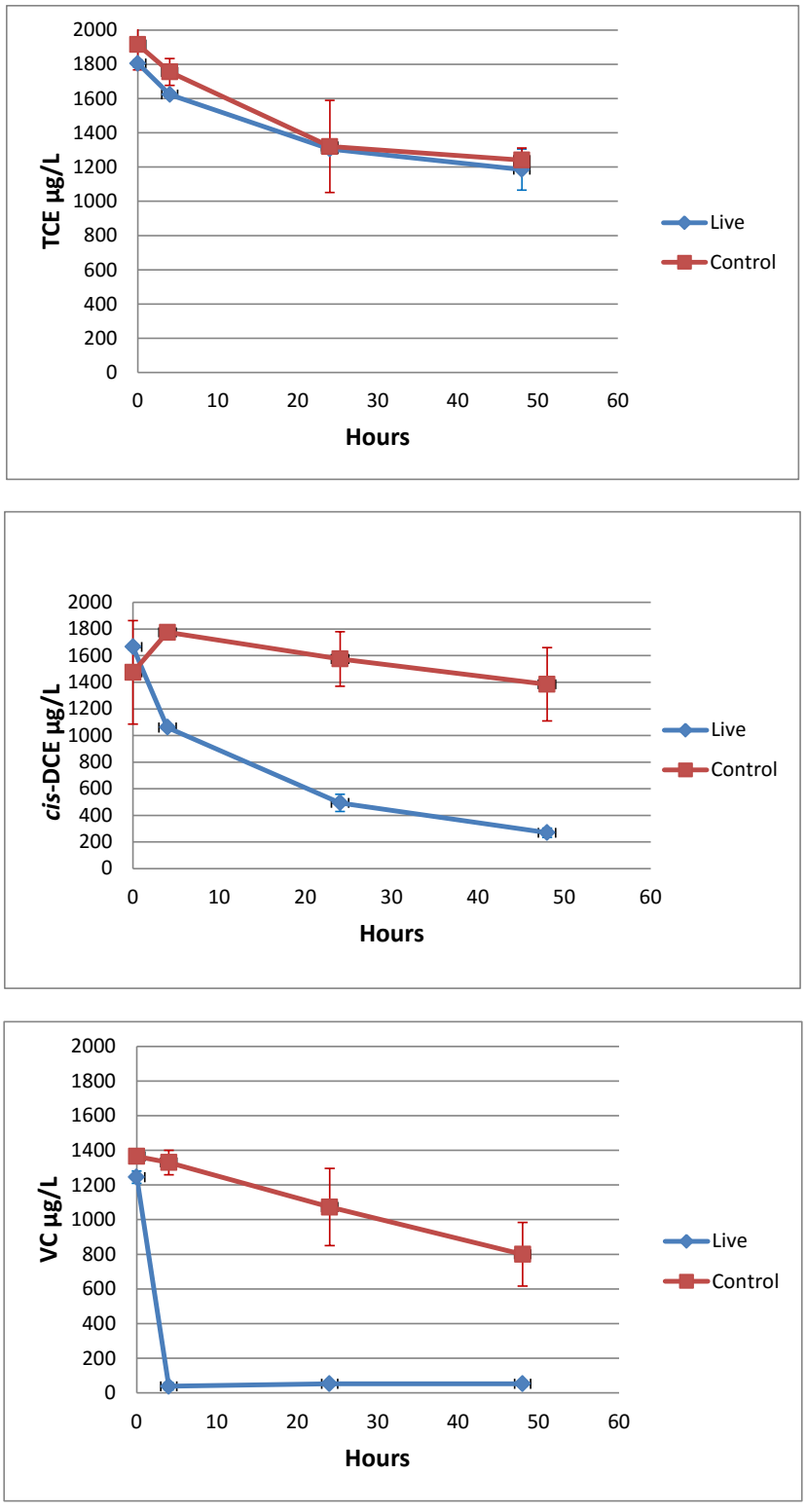


Figure 31. Biodegradation of TCE (top panel), *cis*-DCE (middle panel) and VC (bottom panel) at pH 4 by a IHDIV enrichment culture grown on ethene. Error bars are standard deviations from duplicate samples.

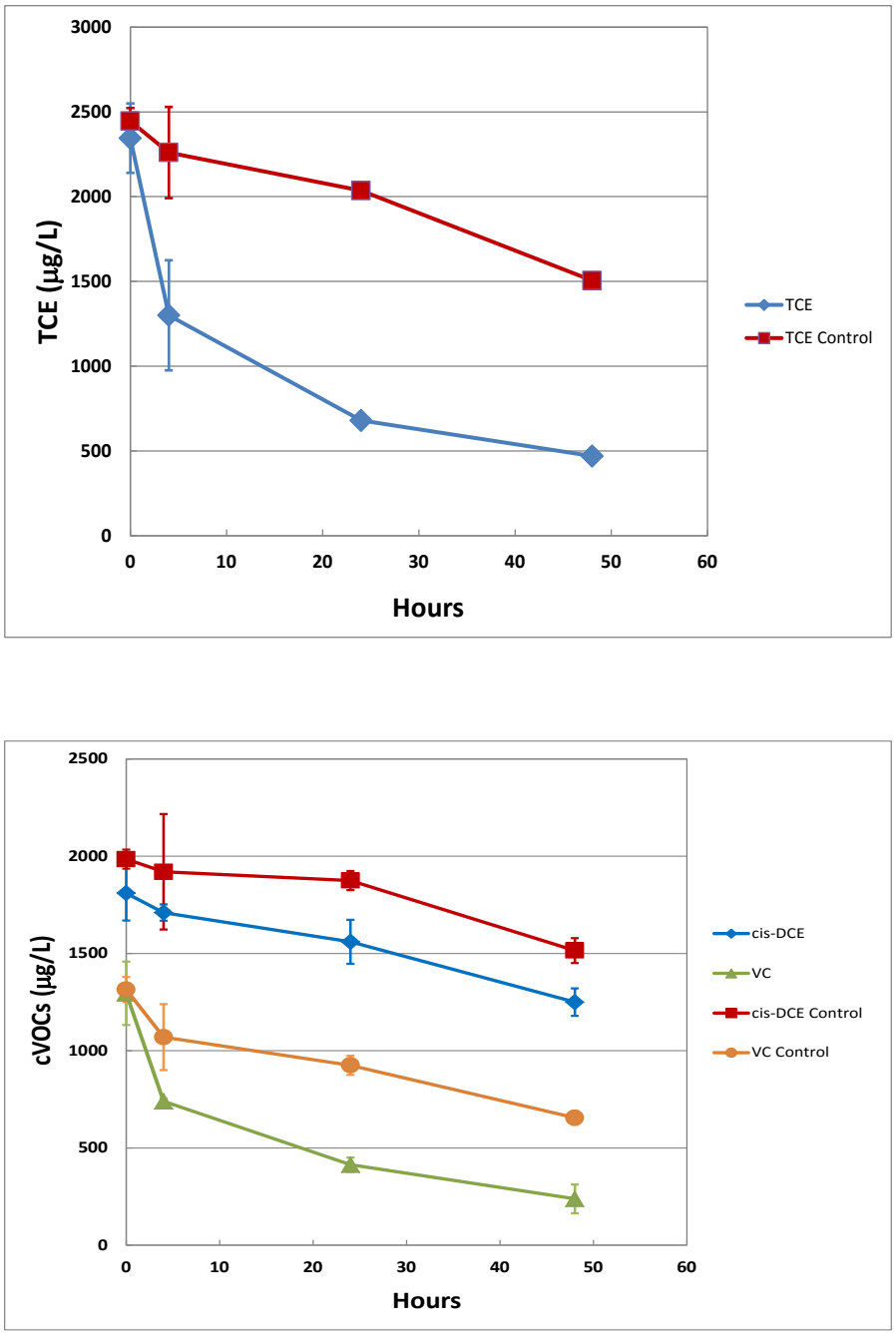


Figure 32. Biodegradation of TCE (top panel), *cis*-DCE (bottom panel) and VC (bottom panel) at pH 4 by a IHDIV enrichment culture grown on methane. Error bars are standard deviations from duplicate samples.

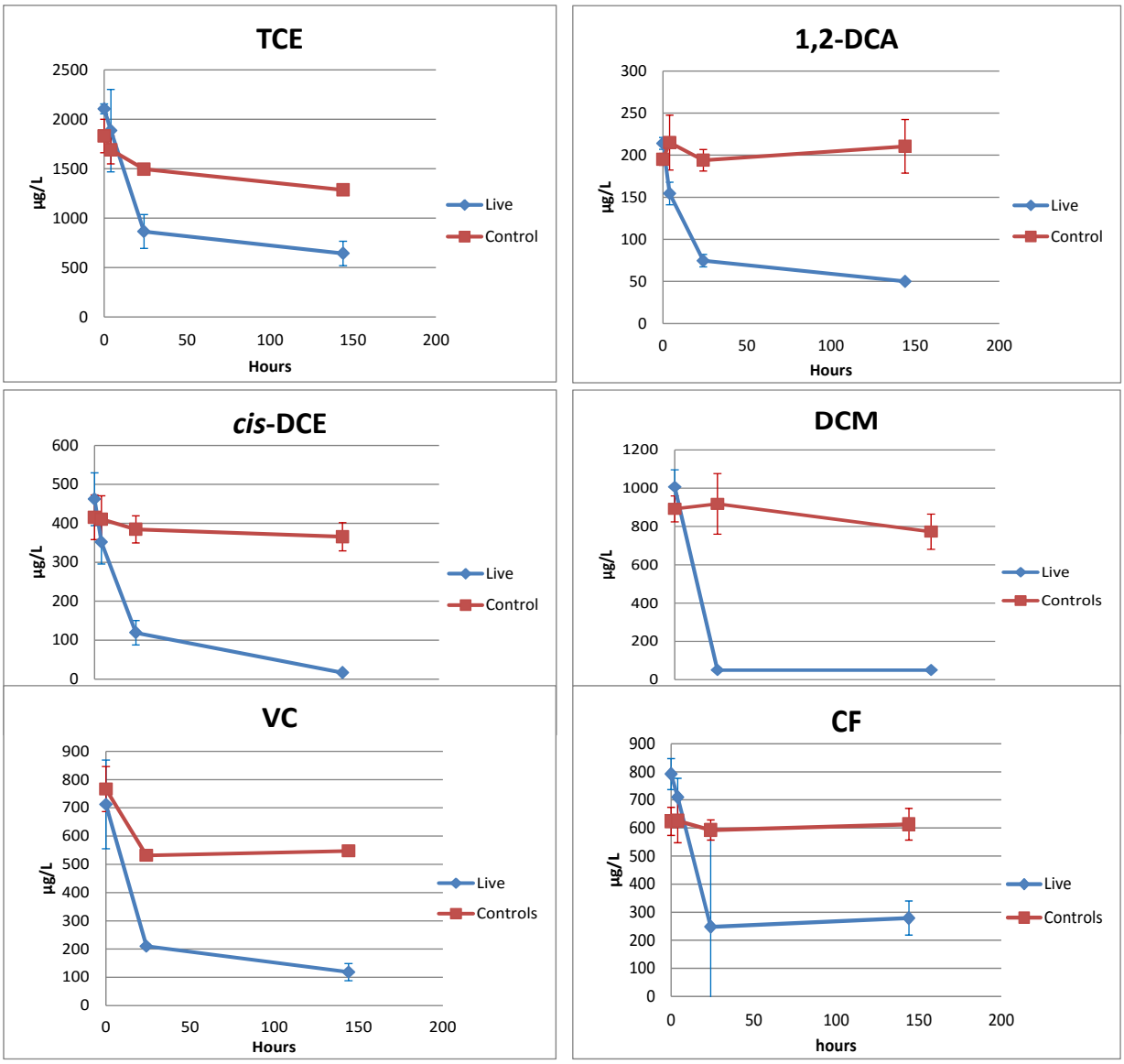


Figure 33. Biodegradation of various cVOCs by a methane-degrading enrichment culture from IHDIV at pH 4 in Cu-free media. Error bars are standard deviations from duplicate samples.

3.3 Microbial Community Characterization and SIP Analysis

3.3.1 Shift of Buoyant Densities of DNA from $^{13}CH_4$-Receiving Microcosms

Samples were collected from Dahlgren microcosms on three occasions after being spiked with ^{13}C-labelled methane and TCE (**Figure 20**). The collected samples were extracted and fractionated as described in the Materials and Methods, Section 2.4. Each DNA fraction was analyzed for the buoyant density (BD) and the abundance of 16S rRNA genes (**Figure 34**). In general, the BD of the peaks shifted towards the right over time. This trend was observed for both NSWC Dahlgren and JBMDL Kirkwood samples. For instance, in the first Dahlgren microcosm sampled (**Figure 34, panel A**), buoyant densities shifted from 1.715g/ml (after 1 day of incubation with $^{13}CH_4$) to 1.718 g/ml (after 3 days of incubation with $^{13}CH_4$). A more pronounced shift was apparent in a replicate microcosm (**Figure 34, panel B**).

For Kirkwood microcosms, subsamples were collected 19 days and 21 days after $^{13}CH_4$ was added (**Figure 12**). The longer incubation time compared to the NSWC Dahlgren microcosms reflected the slower rate of CH_4 consumption in the JBDML Kirkwood samples after the $^{13}CH_4$ was added. The abundance peak in these samples shifted more significantly than that of the Dahlgren samples, which were incubated over a shorter period and may have incorporated slightly less CH_4 into DNA. The peak 16S rRNA shift was from 1.706 (Day 0 sample 31) to 1.718 (Day 21 sample 32). Thus, the DNA fractions corresponding to the peaks, (ones circled in **Figure 34**) were selected for further molecular analyses, including active methanotrophic microbial community structure fingerprinting and bacterial identification.

3.3.2 Identification of Active Methanotrophs in the Microcosms

A total of thirty five 16S rRNA clones, with 24 clones from the NSWC Dahlgren microcosms and 11 clones from the JBDML Kirkwood microcosms, were derived. The phylogenetic relationships between these clones and the known acidophilic methanotrophs are shown in **Figure 35**. The known acidophilic methanotrophs are clustered largely in the in *Alphaproteobacteria*; however, the isolated clones from these sites clustered in *Alphaproteobacteria* and *Gammaproteobacteria*. Two clones from NSWC Dahlgren (D4-XT1-clone2 and D4-XT3-clone7) and 4 clones from JBDML Kirkwood (K5-XT21-clone3, K5-XT21-clone1, K5-XT0-clone3 and K5-XT0-clone7) were in the *Alphaproteobacteria*, but they are not closely related to the known acidophilic methanotrophs. Interestingly, the rest of clones clustered in the *Gammaproteobacteria*, but again were not closely related to known acidophilic methanotrophs. **The results are unexpected but interesting, suggesting that many acidophilic methanotrophs are present in the acidic groundwater and they are more phylogenetically diverse than previously reported. Future work is needed to isolate these methanotrophs to better understanding their abundance and role in biodegradation of contaminants at acidic groundwater sites.**

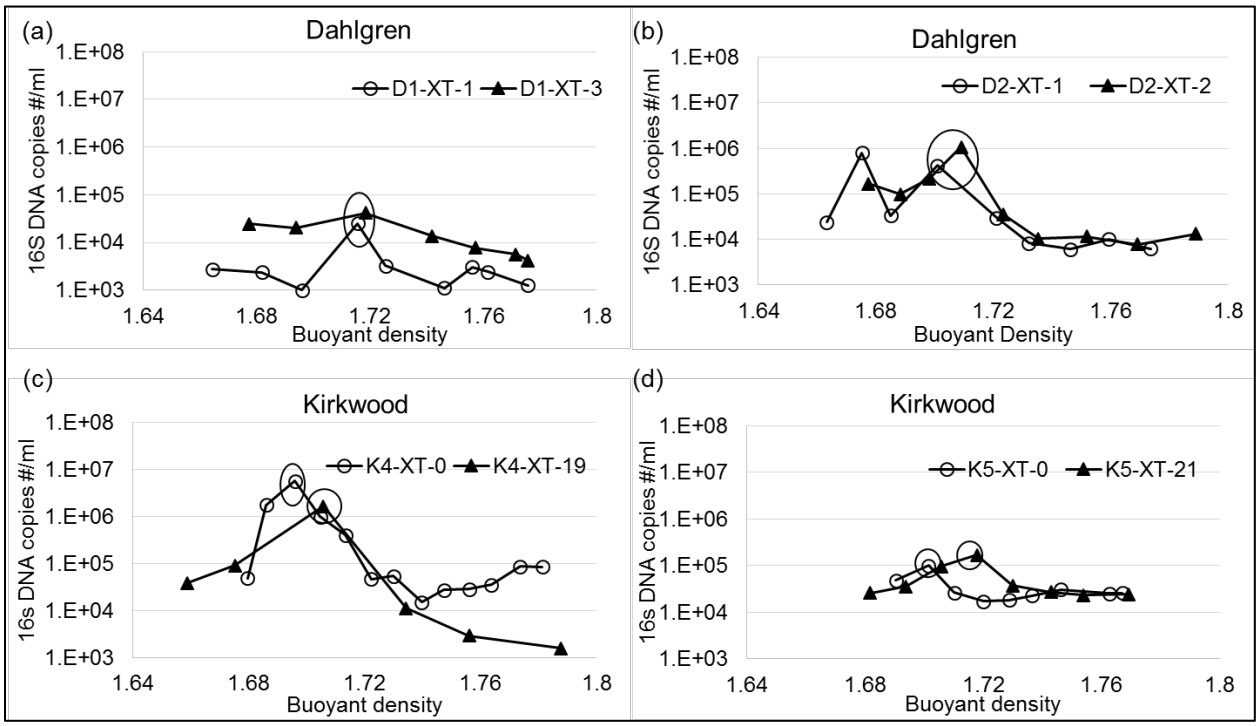


Figure 34. Relative abundance of 16S rRNA gene copies and gradient fractions of day 1 sample (D1-XT-1) and day 3 sample (D1-XT-3) of Dahlgren Microcosm D1 (a); Day 1 sample (D2-XT-1) and day 2 sample (D2-XT-2) of Dahlgren Microcosm D2 (b); Day 0 sample (K4-XT-0) and day 19 sample (K4-XT-19) of Kirkwood Microcosm K4 (c); and Day 0 sample (K5-XT-0) and day 21 sample (K5-XT-21) of Kirkwood Microcosm K5 (d). The codes for sample identities are described in Table 1.

3.3.3 Prevalence of pMMO and sMMO in Acidic Aquifer Microcosms

To understand the distribution of pMMO and sMMO in the microcosms, genomic DNA extracted from all microcosms was screened for the presence of *pmo*A and *mmo*X genes encoding pMMO and sMMO, respectively, using regular PCR. Except for killed controls, either *pmo*A or *mmo*X genes, or both, were detected in live controls and samples (**Table 1**). The *pmoA* gene was detected in many NSWC Dahlgren samples, but only in few of JBDML Kirkwood samples. Interestingly, although *mmoX* (A-B) was not detected on any of the samples, several positive PCR products using the primer set of *mmoX*(LF-LR), which is specific for sMMO in *Methylocella* spp., were observed. *Methylocella* is a distinct taxonomic cluster of acidophilic methanotrophs that only expresses sMMO (Dedysh et al., 2000, 2003, 2004, 2005). Several known sMMO-expressing *Methylocella spp,* including *M. palustris, M. silvestris,* and *M. tundra* can be detected by the *mmoX*(LF-LR) primers. A previous study reported that this primer set can also amplify a small fragment of mmoX gene from other methanotrophs, such as *Methylobacter, Methylocystis or Methylococcus* (Rahman et al., 2011). Thus, the positive results from using this primer set suggested that *Methylocella* and/or *Methylocella* genus-like sMMO were present in the Dahlgren microcosms. Similarly, positive PCR products were also detected in a subset of the Kirkwood microcosms (**Table 1**), suggesting *Methylocella* or *Methylocella*–like methanotrophs were also presence in this aquifer.

Figure 36 shows the diversity of pmoA genes obtained from NSWC Dahlgren and JBDML Kirkwood samples. Interestingly, two different clusters were observed for samples from the two different sites.

PmoA genes derived from Dahlgren aquifer samples show a close relationship to *Methylomonas paludis* MG30 and *Methylocaldum* sp. BFH1 (Islam et al., 2016), which belong to *Gammaproteobacteria* and can only express pMMO (Danilova et al., 2013). Most *pmoA* clones from Dahlgren are clustered in *Gammaproteobacteria*. To our knowledge, *Methylomonas paludis* sp. nov. is currently the only known acidophilic *Methylomonas* expressing pMMO (Danilova et al., 2013). As shown in **Figure 36**, the *pmoA* clones derived from Dahlgren samples are very different from *Methylomonas* species.

The diversity of *mmoX* genes derived is shown in **Figure 37**. The *mmoX* clones derived from Dahlgren samples are more closely related to *Methylococcus*. Different from those observed in Dahlgren samples, three clones from Kirkwood are in clustered in *Alphaproteobacteria* and they are more closely related to *Methylocystis*. Once again, all *mmoX* genes detected in this study are different from those of *Methylocella*, suggesting that other non-*Methylocella* strains might have similar *mmoX* genes.

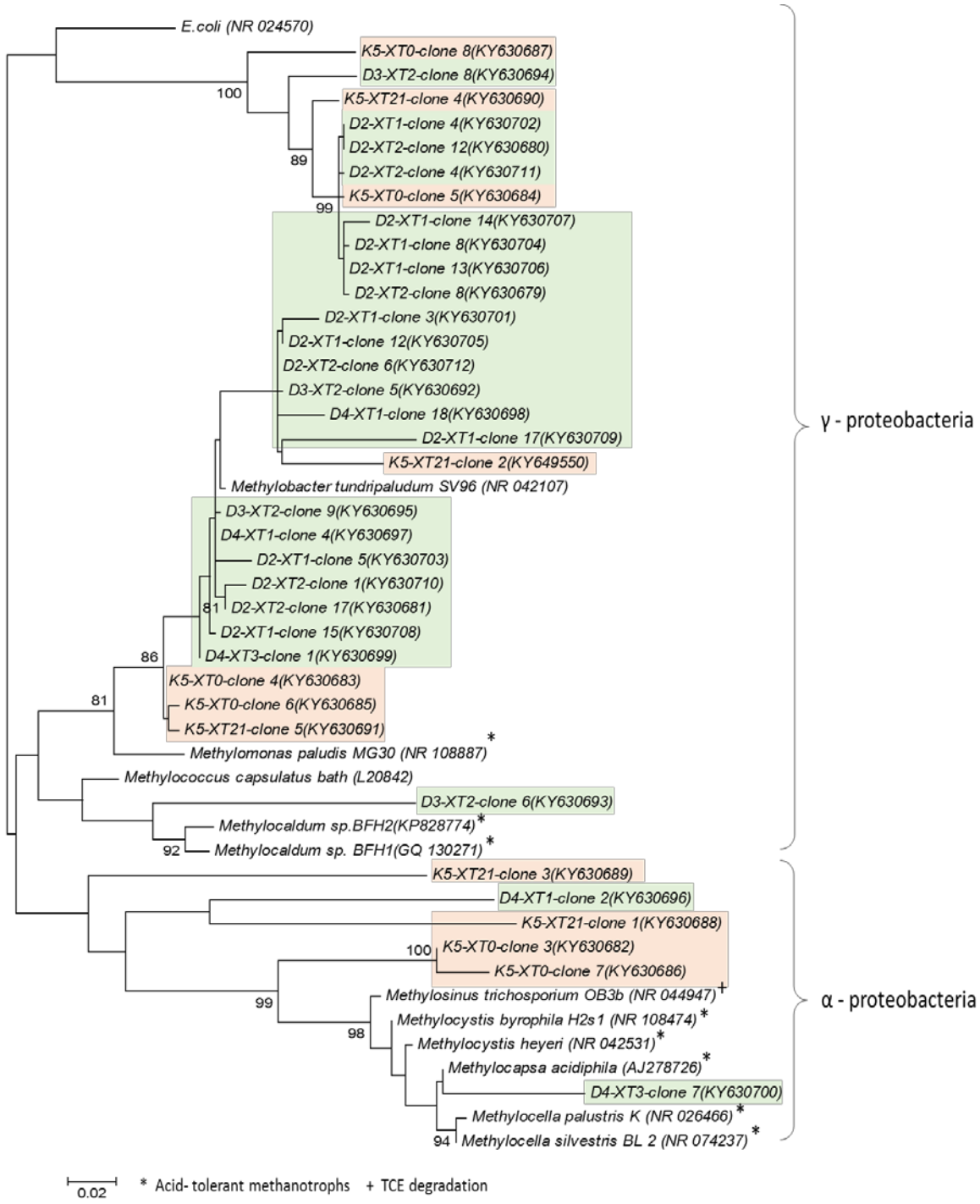


Figure 35. Phylogenetic tree based on 16S rRNA clones derived from microcosms.

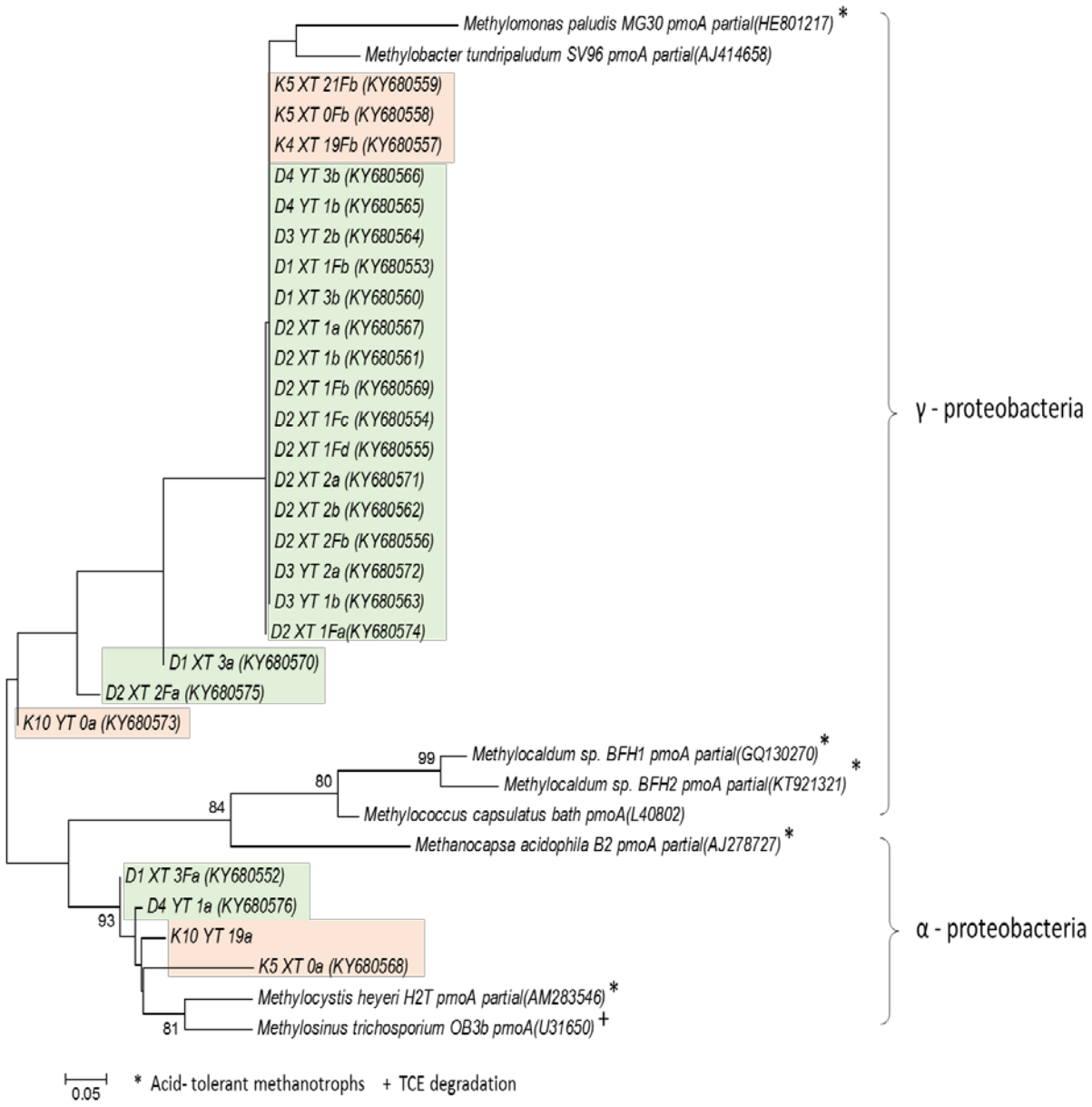


Figure 36. Phylogenetic tree based on *pmoA*. The bootstrap consensus tree inferred from 1000 replicates is presented. The percentage of replicate trees in which the associated sequence clustered together in the bootstrap test is shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.

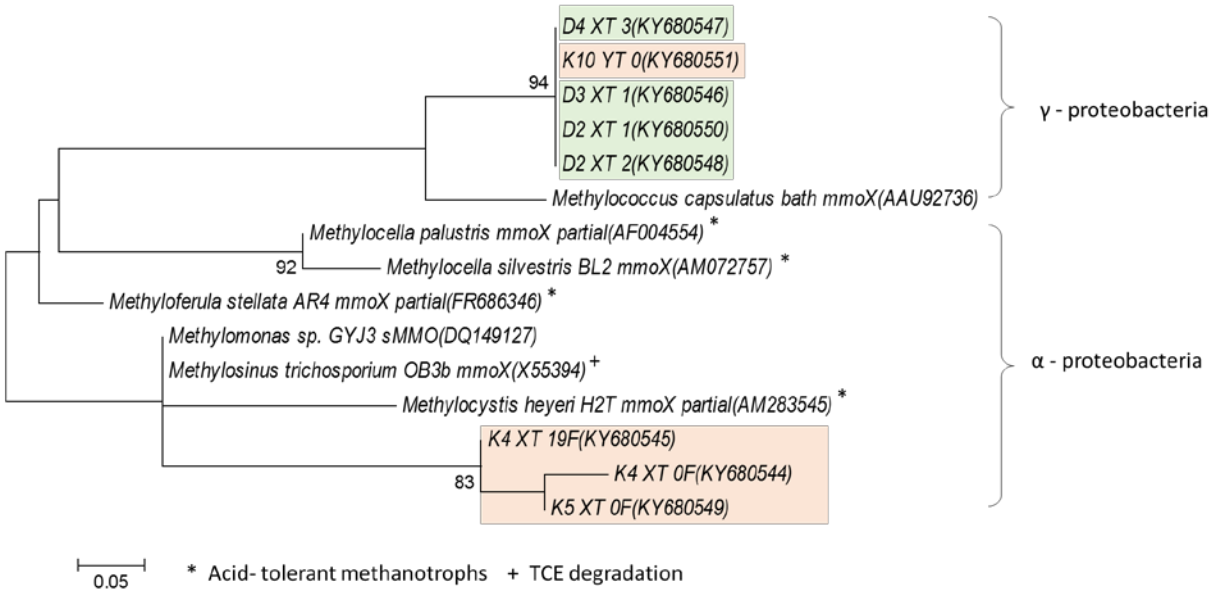


Figure 37. Phylogenetic tree based on *mmoX(LF-LR)*. The phylogenetic relations between samples and known acidophilic methanotrophs based on *mmoX*(LF-LR). The primer set, *mmoX*(LF-LR), was designed to amplify *mmoX* from *Methylocella silvestris* BL2. BLAST results show that the primers also could be used to identify *Methylocella palustris*, *Methylocella tundrea* and *Methylomonas sp.* LC1. The *mmoX* genes in the samples from Dahlgren could be detected by this pair of primers. The phylogenetic tree shows that these genes are not closely related to *Methylocella*, but closely related to *Methylococcus capsulatus Bath*. The results suggested that they may be some uncultured acidophilic methanotrophs belong to other genre.

3.3.4 Acidophilic Microbial Community Structure in TCE-degrading Microcosms

Active acidophilic microbial community structures in acidic TCE-degrading microcosms were examined using real-time-t-RFLP. The DNA fractions corresponding to the peaks (circled) in **Figure 34** were used as templates for the analysis. The profiles derived from these templates can be considered as active acidophilic methanotrophic communities in the microcosms. As shown in **Figure 38**, several ribotypes (represented as t-RFs), t-RFs = 97,103,105,114,117 bp, were observed in all Dahlgren and Kirkwood samples. T-RFs = 77 and 83 bp were detected in the Dahlgren samples, while t-RF = 99 bp was detected only in the Kirkwood samples. The *in-silico* analysis of thirty-five 16S rRNA sequences derived from microcosms receiving $^{13}CH_4$ (as shown in **Figure 35**), the theoretical fragments of ten clones correspond to three different ribotypes, i.e., t-RFs=83, 99, 105.

The t-RF of 105 bp observed in all the profiles corresponds to the theoretical t-RF of several known methanotrophs, including *Methylomonas, Methylocystis, Methylosinus,* and *Methylocella,* suggesting these methanotrophs might be present in acidic groundwater. *Methylomonas* can only express pMMO, while *Methylocella* can only express sMMO, *and Methylocystis* and *Methylosinus* can express both pMMO and sMMO depending on growth conditions (Ul-Haque et al., 2015; Lee et al., 2006). *Methylocella palustris* (Dedysh et al., 2000), *Methylocella silvestris* (Dunfield et al., 2003), *Methylocella tundrea* (Dedysh et al., 2004), *Methylocystis broyphila* (Belova et al., 2013), *Methylosinus* and *Methylomonas palustis* (Danilova et al., 2013) were isolated from environments at pH lower than 5. Strains BFH1 and BFH2 are thermophilic methanotrophs within the genera *Methylococcus* (Islam et al., 2016) belonging to the *Gammaproteobacteria*. Strains BFH1 and BFH2 optimally grow at pH 5.5 to 6.0 and their theoretical t-RF is 83 bp. *Methylococcus capsulatus* Bath is a well-studied Type I methanotroph that can tolerate low pH; it also has a theoretical T-RF of 83 bp. Therefore, as the fragment of t-RF = 83 bp was observed in community profiles of NSWC Dahlgren samples, it is likely that BFH1 and BFH2-like acidophilic methanotrophs are present in the NSWC Dahlgren aquifer.

Table 1. Detection of *mmoX* and *pmoA* genes in microcosm samples.

Microcosm Setup and naming				Sample name	*mmoX* gene		*pmoA* gene	
Groundwater [Dahlgren (D) or Kirkwood (K)]	Microcosm #	Substrate amended [$^{13}CH_4$ (X); $^{12}CH_4$ (Y); TCE (T)]	Sampling Time [day]		*mmoX*L (F-R)	*mmoX* (A-B)	A189-mb661	A189-A682
D	1	XT	1	D1-XT-1	-	-	-	-
			3	D1-XT-3	-	-	+	+
D	2	XT	1	D2-XT-1	+	-	+	+
			2	D2-XT-2	+	-	+	+
D	3	YT	1	D3-YT-1	+	-	+	+
			2	D3-YT-2	+	-	+	+
D	4	YT	1	D4-YT-1	-	-	-	+
			3	D4-YT-3	-	-	+	+
D	5	XT	1	D5-XT-1	-	-	-	-
			3	D5-XT-3	-	-	-	-
D	6	XT	1	D6-XT-1	-	-	-	-
			2	D6-XT-2	-	-	-	-
D	7	YT	1	D7-YT-1	-	-	-	-
			2	D7-YT-2	-	-	-	-
D	8	YT	1	D8-YT-1	-	-	-	-
			3	D8-YT-3	-	-	-	-
K	1	X	0	K1-X-0	-	-	-	-
			12	K1-X-12	-	-	-	-
K	2	X	0	K2-X-0	-	-	-	-
			12	K2-X-12	-	-	-	-
K	3	X	0	K3-X-0	-	-	-	-
			12	K3-X-12	-	-	-	-
K	4	XT	0	K4-XT-0	+	-	-	-
			19	K4-XT-19	+	-	+	-
K	5	XT	0	K5-XT-0	+	-	+	+
			21	K5-XT-21	+	-	+	-
K	6	XT	0	K6-XT-0	-	-	-	-
			21	K6-XT-21	-	-	-	-
K	7	Y	0	K7-Y-0	-	-	-	-
			12	K7-Y-12	-	-	-	-
K	8	Y	0	K8-Y-0	-	-	-	-
			12	K8-Y-12	-	-	-	-
K	9	Y	0	K9-Y-0	-	-	-	-

			12	K9-Y-12	-	-	-	-
			0	K10-YT-0	-	-	+	-
K	10	YT	19	K10-YT-19	-	-	+	-
			0	K11-YT-0	-	-	-	-
K	11	YT	21	K11-YT-21	-	-	-	-
			0	K12-YT-0	-	-	-	-
K	12	YT	21	K12-YT-21	-	-	-	-
K	13	X	0	K13-X-0	-	-	-	-
			12	K13-X-12	-	-	-	-
K	14	X	0	K14-X-0	-	-	-	-
			19	K14-X-19	-	-	-	-
K	15	X	0	K15-X-0	-	-	-	-
			21	K15-X-21	-	-	-	-
K	16	Y	0	K16-X-0	-	-	-	-
			12	K16-X-12	-	-	-	-
K	17	Y	0	K17-X-0	-	-	-	-
			19	K17-X-19	-	-	-	-
K	18	Y	0	K18-X-0	-	-	-	-
			21	K18-X-21	-	-	-	-

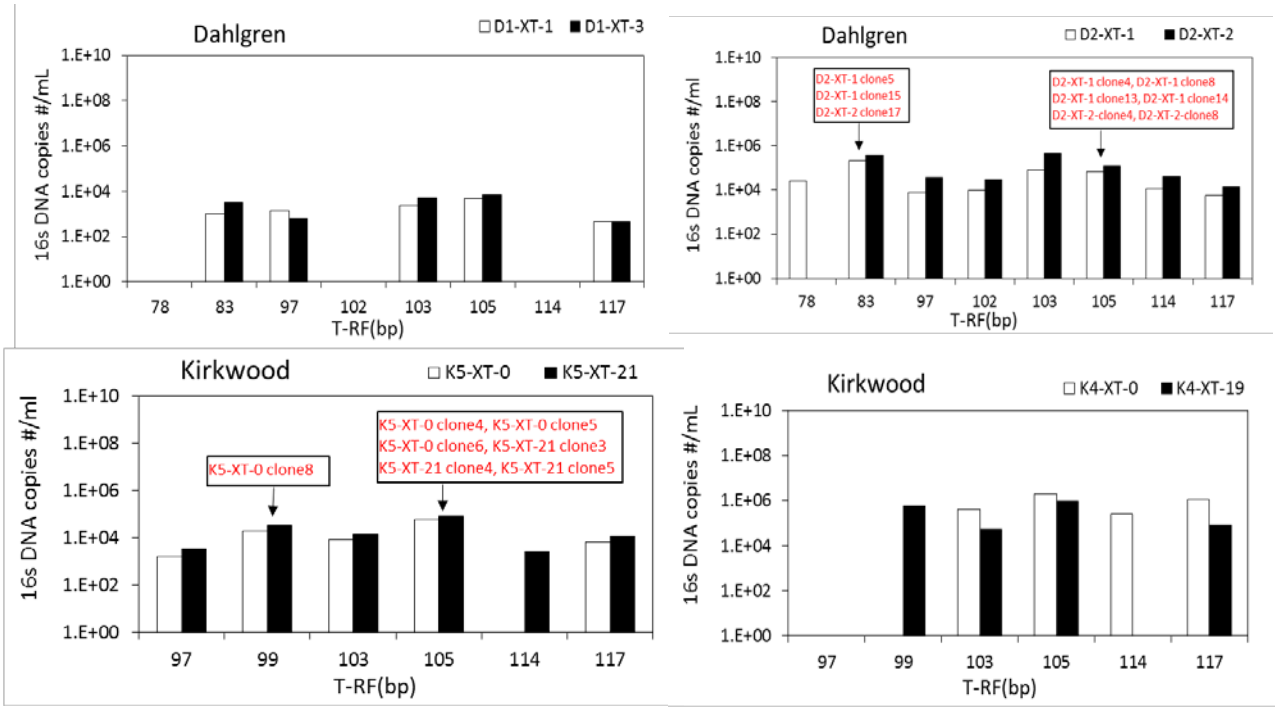


Figure 38. Changes of microbial community structure in TCE-degrading methanotrophic microcosms containing acidic groundwater from NSWC Dahlgren (top panels) and JBMDL Kirkwood (bottom panels).

3.4 Flow-Through Aquifer Columns

The influent and effluent groundwater pH for Column 1 (C1; methane) averaged 4.9 ± 0.2 SU and 4.9 ± 0.2 SU, respectively. Influent and effluent pH for column 3 (C3; no methane) averaged 4.1 ± 0.2 SU and 4.7 ± 0.2 SU, respectively. Thus, methane oxidation had little influence on pH across the column. Methane consumption across both C1 and C2 was observed during the first 50 days of operation, with more rapid degradation occurring in C1 than C2 (**Figure 39**). C2 was placed in series with C1 on Day 138, so all data from this point to the end of the study on Day 212 are plotted as C1 results. For C1, from ~ Day 50 to the end of the study, influent methane averaged 400 ± 100 µg/L and effluent methane averaged 3 ± 4 µg/L. Thus, methane was clearly consumed across the C1, both before and after this column was linked in sequence with C2 to increase hydraulic residence time. The fact that influent and effluent methane concentrations were similar during the first few weeks of the study for both columns indicates that methane loss was biological rather than due to volatilization or another abiotic process. Influent dissolved oxygen (DO) in C1 averaged 10.6 ± 0.7 mg/L and effluent DO averaged 8.1 ± 0.7 mg/L. Similarly, in C2 during the first 138 days, influent DO averaged 10.6 ± 0.5 mg/L and effluent DO averaged 8.6 ± 0.6 mg/L. In the control column (C3), the same values were 10.6 ± 0.5 mg/L for the influent and 9.3 ± 0.6 mg/L for the effluent. Thus, consistent with oxidation of methane, there was a greater decrease in DO across C1 than C3 during the study. C2 was only operated independently for 138 days, and good methane consumption was only observed during the last month or so of independent operation.

Clearly, the data from C1 and C2 indicate the presence of methanotrophs in the aquifer solids and the ability of these methanotrophs to consume methane at ~ 400 µg/L to < 5 µg/L over a significant period of time. These data are consistent with both the microcosm and enrichment culture data from the JBMDL Kirkwood aquifer samples. The TCE data from C1 and C3 are plotted in **Figure 40** as % removal of TCE. The average influent TCE concentration to the C1 and C3 columns combined was 160 ± 80 µg/L over the course of the study. Overall the percent removal in C1 was ~ 10% greater than that in C3 over the course of the study. This percentage did not change markedly when the column residence time was doubled (i.e., when C1 and C2 were run in sequence) or when Cu was added to the influent to stimulate pMMO rather than sMMO. Overall, the percent removal was greater in the methane-fed column(s) than in the control column on 72% of the sampling events.

The reason that TCE was not biodegraded more efficiently in the columns may have several explanations, the most likely of which are (1) lack of overall microbial biomass due to the low methane concentrations applied, combined with the relatively short residence time in the columns (24 hr to 48 hr); and/or (2) the variability in the influent methane and TCE concentrations over time, particularly considering the potential for inhibition of residual methane on TCE biodegradation. It is possible, even likely that the percentage of TCE removed would have increased in the columns over time as methanotrophs biomass increased, but in that this was a limited scope project, further operation of the columns was not possible. Similarly, dissolving three different gases (TCE, methane, oxygen) to desired and consistent concentrations in groundwater within Tedlar bags over time is difficult, and variability, particularly affecting the methane/TCE ratio may have influenced the data. In future studies, it would be interesting to design an experimental system where very low methane and TCE (or *cis*-DCE and VC) concentrations can be measured over a long time period to better evaluate the role of methanotrophs in cVOC degradation in low pH environments under more realistic *in situ* conditions at many sites. A similar study

was conducted by Gossett (2010) to evaluate aerobic VC biodegradation in environments with very low oxygen concentrations over a long timeframe. Similar work is ongoing in the laboratory of D. Freedman at Clemson University, but ^{14}C-TCE is being utilized to improve the detection of TCE conversion via aerobic processes in site samples (*personal communication, D. Freedman*). These studies do not specifically involve acidic aquifer samples.

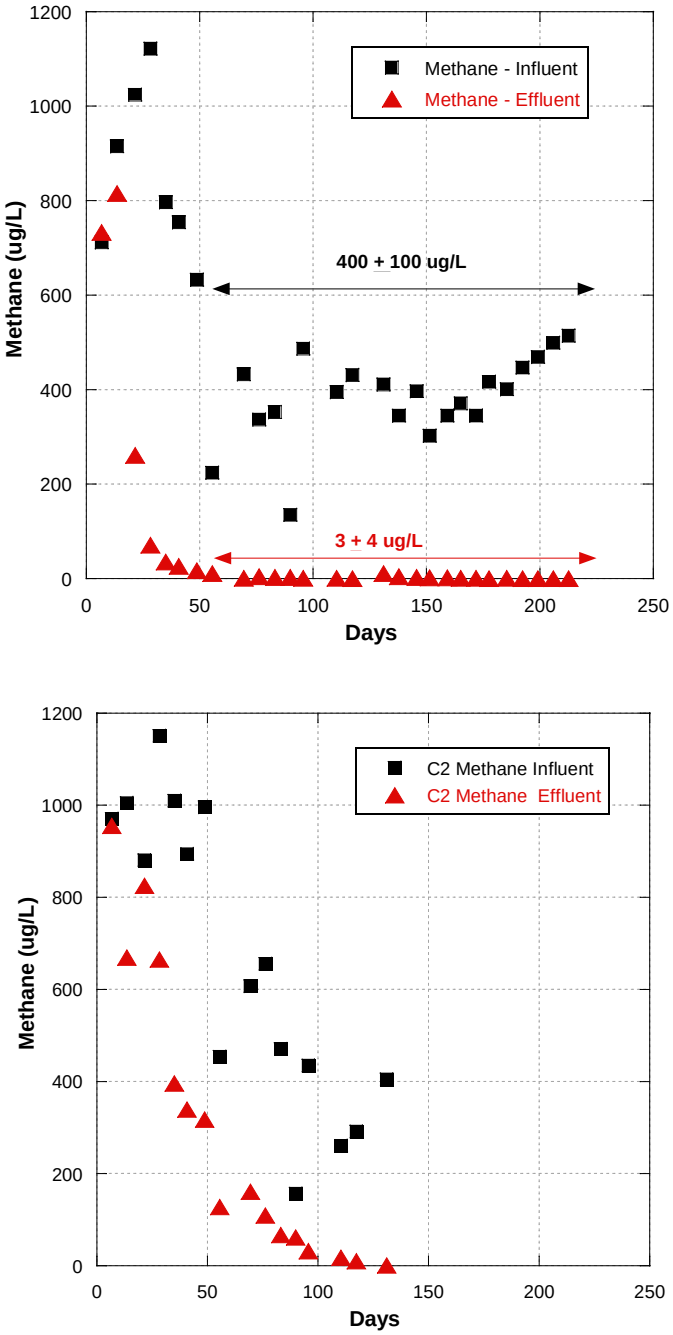


Figure 39. Concentration of methane the influent and effluent groundwater for column 1 (C1) and column 2 (C2).

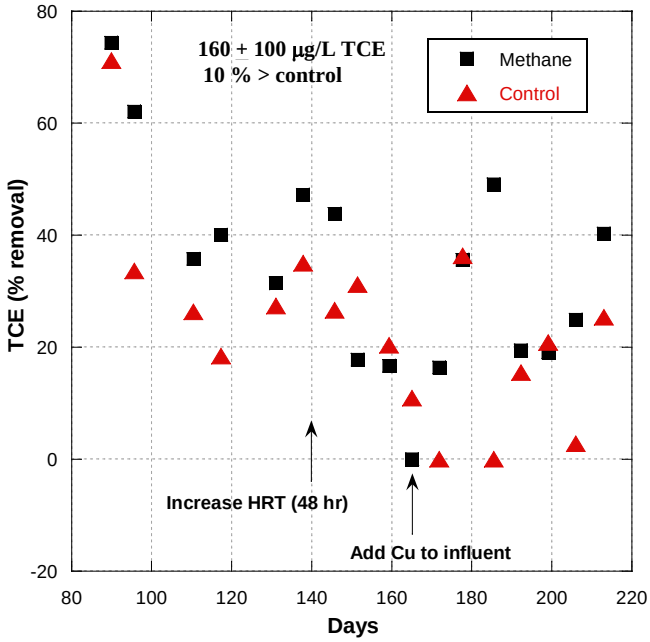


Figure 40. Percent removal of TCE in C1 (methane) compared to C 2 (control).

4.0 CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

The main focus of this project was to determine the extent to which acidophilic methanotrophs may contribute to the biodegradation of cVOCs in low pH groundwater aquifers. Our objectives were to (1) determine whether methanotrophs exist in acidic groundwater aquifers and are capable of degrading methane and cVOCs, with a specific focus on TCE; and (2) identity key methanotrophs and/or methane-oxidizing genes present in acidic groundwater systems using advanced molecular techniques. To achieve these objectives, microcosms were initially prepared with samples from three different acidic aquifers to determine whether the addition of methane and air could be used to stimulate native methanotrophs to biodegrade TCE and other cVOCs. Ethane and ethene were also tested as cometabolic substrates at one of the sites. Small samples of aquifer solids from each site were incubated in growth media with methane (as well as with ethane or ethene in a few cases) as a sole carbon source in order to enrich active indigenous communities. In addition, characterization of the methanotrophic communities in the acidic aquifers was conducted using SIP and probing for *pmoA* and *mmoX* genes in samples, which code for the two common enzymes used by methanotrophs to convert methane to methanol and also to oxidize cVOCs.

Methane consumption and degradation of cVOCs was observed in acidic aquifer samples collected from all three DoD sites. Moreover, methanotrophic enrichment cultures capable of degrading TCE and a number of other cVOCs at pH 4 were isolated from two of the three sites. These data clearly show that methanotrophs commonly exist in acidic groundwater aquifers and that they have the potential to biodegrade cVOCs at low pH. Cultures capable of degrading cVOCs at pH 4 after growth on ethane or ethene also were enriched. Although degradation of only a small subset of chlorinated ethenes and ethanes by the various enrichment cultures was evaluated, the data indicate differences in the cVOCs degraded among the acidophilic enrichment cultures and between some of these cultures and the more widely studied neutrophilic methanotrophs. Further study is warranted to better understand the selectivity of the presumptive sMMO or pMMO enzymes catalyzing reactions under acidic conditions.

SIP analysis of active microcosms from the two sites tested showed that a variety of methanotrophs incorporated ^{13}C into their DNA (from added $^{13}CH_4$). A total of thirty five 16S rRNA clones were derived and identified. The clones clustered largely in the *Alphaproteobacteria* and *Gammaproteobacteria* but were not closely related to known acidophilic methanotrophs. The results are unexpected but interesting, suggesting that many methanotrophs are present in the acidic groundwater and that they are more phylogenetically diverse than previously reported. Future work is needed to isolate these acidophilic methanotrophs to better understanding their microbial physiology, degradative ability toward cVOC, abundance, and role in biodegradation of other contaminants at acidic groundwater sites.

Overall, this project showed that diverse acidophilic methanotrophs are present in groundwater aquifers, and many of them are previously unknown. Most of all, these organisms can potentially be important in degradation of TCE and other cVOCs. There is very little research on this group of organisms in acidic groundwater, their potential contribution to natural attenuation of cVOCs, or the possibility of enhancing their activity. As reductive dechlorination does not generally occur at low pH, aerobic cometabolism may be particularly important as a remedial mechanism for cVOCs in acidic aquifers. The microcosm and

enrichment culture data also indicated the presence of ethane- or ethane-oxidizing organisms that were capable of growing at low pH and degrading cVOCs. Again, the potential for using these cometabolic substrates (which are often formed during reductive dechlorination of cVOCs) to stimulate also has received little study to date.

Based on the initial results of this Limited Scope Study, there are several areas that we believe warrant additional investigation including (1) measuring the activity and degradation kinetics of acidophilic methanotrophs at low methane and cVOC concentrations as may be observed in dilute cVOC plumes that are problematic for DoD; (2) assessing the potential for acidophilic methanotrophs from groundwater to biodegrade cVOCs after growth on substrates other than methane (e.g., small fatty acids, alcohols), as several methanotrophs recently been described that are facultative; (3) further assessing the suite of cVOCs that may be susceptible to degradation by acidophilic methanotrophs, to include chlorinated methanes, ethanes, ethenes, and whether activity is culture-specific; (4) evaluating key factors contributing to differences in cVOC degradation among sites and/or strains (e.g., effect of dissolved metals or other co-contaminants, extent of substrate inhibition, pH optima and extremes); and (5) more clearly identifying the types of MMO enzymes that are active at low pH, determining how they differ from those of neutrophilic bacteria, and developing appropriate primer sets to detect and quantify them via qPCR as a measure of degradative potential in acidic aquifers.

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